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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Russell & Connell	§	**ART UNIT: 1632**
FILED: April 3, 2001	§	
SERIAL NO.: 09/825,105	§	**EXAMINER: Li, Quan**
FOR: Chimeric Antigen-Enterotoxin Mucosal Immunogens	§	**DOCKET: D6321**

MS Appeal Brief - Patents
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313

TRANSMITTAL OF APPEAL BRIEF
AND CERTIFICATE OF MAILING UNDER 37 CFR 1.8

Dear Sir:

Enclosed please find three originals of the Appeal Brief for the above-referenced patent application.

The Commissioner is hereby authorized to charge Deposit Account No. 07-1185 in the total amount of $165 for the appeal fee and any additional fee that may be required. Please credit any overpayment or debit any underpayment to Deposit Account 07-1185.

I hereby certify under 37 CFR 1.8 that the following correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to MS Appeal Brief, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. Please return the enclosed postcard acknowledging receipt of this correspondence.

Respectfully submitted,

Date: Dec 5, 2003

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Image AF/GP1632

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Mail Stop Appeal Brief - Patents
Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313

ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on October 7, 2003. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the UAB Research Foundation.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Originally claims 1-29 were filed with this Application. Claims 4-5 and 7-23 were canceled by amendment. The pending claims 1-3, 6, 24-29 are being appealed of which claims 1, 24 and 27 are an independent claims.

IV. STATUS OF AMENDMENTS

Subsequent to the final rejection mailed May 6, 2003, Applicants submitted a Response After Final which canceled claims 9-23. All pending claims are shown in Appendix A.

V. SUMMARY OF THE INVENTION

The present invention provides methods of inducing immune responses by recombinant antigen-enterotoxin chimeric mucosal immunogens comprising the A2/B subunits of heat-labile type II toxins (see Abstract). The enzymatically active A1 subunit of heat-labile type II toxin was replaced with an immunogen such as the saliva-binding region (SBR) from the streptococcal adhesin AgI/II (page 6, lines 7-15). Intranasal immunization of BALB/c mice with the chimeric proteins induced significantly higher plasma and mucosal anti-SBR IgA and IgG antibody responses (page 40, lines 3-9).

The present invention indicates that heat-labile type II toxins (LT-IIa and LT-IIb) and cholera toxin (a type I heat-labile

enterotoxin) induced secretion of different cytokines from anti-CD3-stimulated human peripheral blood mononuclear cell cultures (page 54, lines 4-18), possibly due to differential effects on CD40L expression and IL-12 secretion (page 48, line 17 to page 49, line 5). Accordingly, chimeric immunogens comprising the A2/B subunits of heat-labile type II toxins may possess unique immunomodulatory properties on $CD4^+$ T cells due to secretion of Th1 cytokine (page 40, lines 19-21).

The present invention also indicates an advantage for chimeric immunogens based on heat-labile type II toxins in that these chimeric proteins induced substantially lower antibody responses to the enterotoxin components of the immunogens. This feature may permit repeated administration of heat-labile type II toxin-based chimeric immunogens without the loss of immunogenicity due to pre-existing antibodies against the enterotoxin (page 41, lines 1-6).

VI. ISSUES

35 U.S.C. §103

Whether claims 1-3, 6 and 24-29 are obvious over **Toida** et al. (*Infect. Immunity*, 1997) in view of **Rappuoli** et al. (*Immunol. Today*, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 1989; *Vaccine*, 1990) and **Connell** et al. (*Immuol. Lett.*, 1998; *Infect. Immunity*, 1992) under 35 U.S.C. §103(a).

Whether claims 1-3, 6 and 24-29 are obvious over **Russell** et al. (U.S. patent no. 6,030,624) in view of **Rappuoli** et al. (*Immunol. Today*, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 1989; *Vaccine*, 1990) and **Connell** et al. (*Immuol. Lett.*, 1998; *Infect. Immunity*, 1992) under 35 U.S.C. §103(a).

VII. GROUPING OF CLAIMS

The rejected claims do stand or fall together.

VIII. ARGUMENTS

Rejection Under 35 U.S.C. §103

In the Advisory Action mailed August 26, 2003, the Examiner maintained the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) as being unpatentable over **Toida** et al. (Infect. Immunity 65:909, 1997) in view of **Rappuoli** et al. (Immunol. Today 20:493, 1999), and further in view of **Schodel** et al. (Infect. Immunity, 57:1347, 1989; Vaccine 8:569, 1990) and **Connell** et al. (Immuol. Lett. 62:117, 1998; Infect. Immunity 60:1653, 1992). Applicant respectfully requests that this rejection be reversed.

The present invention is drawn to methods of inducing cellular immune response or Th1 immune response (i.e., T cell-mediated immunity) by a fusion protein comprising an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin.

In contrast, **Toida** et al. describe a method of inducing humoral (antibody) and cellular (T helper cell) immune responses using a chimeric immunogen comprising an antigen fused to the A2 and B subunits of cholera toxin (a type I heat-labile enterotoxin). **Rappuoli** et al. describe the the structure and mucosal

adjuvanticity of cholera and *E. coli* heat labile enterotoxin. **Schodel** et al. describe inducing T cell immune responses (but not humoral immune response) against an antigen using a fusion protein which consists of an antigen fused to the B subunit of *E. coli* heat labile enterotoxin.

Connell et al. (1992) describe the structural characterization of hybrid toxins produced by assembly of A and B polypeptides from type I and type II heat labile enterotoxins. **Connell** et al. (1998) describe the induction of humoral immune response upon co-administration of a weak immunogen and a type II heat labile enterotoxin.

Rappuoli et al. and **Schodel** et al. only teach heat labile enterotoxins in general without discussing any similarities and differences between type I and type II heat labile enterotoxins. The Examiner acknowledges that **Rappuoli** et al. and **Schodel** et al. do not distinguish type II from type I heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4). The Examiner argues, however, the gap in teaching on type II heat-labile enterotoxin can be filled by **Connell** et al. The Examiner's rejection is based on the assertion that **Connell** et al. compensate for the lack of teaching on type II heat labile enterotoxins in **Rappuoli** et al. and **Schodel** et al.

Accordingly, Applicant focuses the following discussion on **Connell** et al.

Asserting that **Connell** et al. (1992) teach structural compatibility between type I and type II heat labile enterotoxins, and **Connell** et al. (1998) teach type I and type II heat labile enterotoxins can be used as mucosal adjuvants, the Examiner concludes that apparently there is not much differences in adjuvant properties between type I and type II heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4). Hence, according to the Examiner, it would have been obvious to replace the type I heat labile enterotoxin in **Toida** et al. with a type II heat labile enterotoxins. Applicant submits that the Examiner's broad assertion of same adjuvant properties between type I and type II heat labile enterotoxins is not supported by the cited references and lacks a scientific basis.

There are significant structural differences between type I and type II heat labile enterotoxins. Within the A and B polypeptides of the enterotoxins, only the A1 fragments are homologous between type I and type II heat labile enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 13-20). The A2 fragments are much less homologous than the A1 fragments, and

the B polypeptides of type I enterotoxins have little or no significant homology with those of type II enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 20-24).

The Examiner acknowledges there is significant structural differences between type I and type II heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4; Advisory Action mailed August 26, 2003). The Examiner asserts, however, the **Connell** et al. references were cited to show type I and type II heat labile enterotoxins possess similar biological activities in spite of the structural differences (Advisory Action mailed August 26, 2003, citing first three rows of Table 2 in **Connell** et al., 1992).

Applicant submits that the term "similar biological activities" needs to be clarified in terms of what were taught in **Connell** et al. **Connell** et al. (1992) disclosed biological activities in terms of toxicity and structural integrity which was determined via recognition by enterotoxin subunit-specific antibodies (Table 2, **Connell** et al., 1992). **Connell** et al. (1998) taught induction of humoral immune response. Hence, in view of the significant structural differences between type I and type II heat labile enterotoxins, **Connell** et al. (1992, 1998) teach similar properties in toxicity, structural integrity and the ability to induce humoral

immune response. However, biological functions on toxicity, structural integrity and induction of humoral immune response do not provide any scientific basis for predicting the likelihood of inducing cellular or T cell-mediated immune response by type II heat labile enterotoxins. **Connell** et al. or other references cited by the Examiner do not provide any teaching or guidance on how to relate the properties of toxicity, structural integrity and induction of humoral immune response to the capacity of inducing cellular immune response. In other words, even though type I and type II heat labile enterotoxins have similar properties in toxicity, structural integrity and induction of humoral immune response, that does not mean these two types of enterotoxins would have similar properties in the induction of cellular immune response because the recited similar activities do not have direct and logical relationship to the capacity of inducing cellular immune response.

A person having ordinary skill in this art would readily recognize that toxicity and immunity are two distinct and separate areas of biological activities. Regarding the teaching on induction of humoral immunity, it is important to recognize the distinct features of humoral immunity (antibody immune response) vs. cellular immunity (T cell-mediated immune responses). The distinction

between humoral immunity and cellular immunity is significant and important. It is a basic concept in immunology that these two kinds of immune responses are stimulated by different immunogenic peptides (Class I MHC-restricted peptides vs. Class II MHC-restricted peptides) and are mediated by different immune effector cells (T cells vs. B cells). Hence, the parameters for the induction of humoral immunity are different from that for the induction of cellular immunity. One of ordinary skill in the art would readily recognize that parameters for the induction of humoral immunity are mostly, if not all, not suitable or applicable for the induction of cellular immunity. Therefore, the teaching on the induction of humoral immunity as disclosed in **Connell** et al. (1998) does not shed any light on whether an immunogen comprising a type II heat labile enterotoxin can induce cellular immune responses. Accordingly, the issue of whether an immunogen (in the present case, a type II heat labile enterotoxin) can induce an immune response (in the present case, cellular immunity) has to be resolved by actual experimentation.

The need for empirical experiments is also highlighted by the significant structural differences between type I and type II heat labile enterotoxins. The present invention relates to induction of

cellular immune response by a chimeric antigen comprising the A2 and B subunits of type II heat-labile enterotoxin. In contrast, the cited prior art references teach induction of cellular immune response by a chimeric antigen comprising the A2 and B subunits of type I heat-labile enterotoxin (**Toida** et al.). As discussed above, the A2 and B subunits of type I heat-labile enterotoxin have little or no significant homology with those of type II enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 20-24). The cited prior art references do not provide any logical or scientific reasoning that would have indicated to a person having ordinary skill in this art that the A2 and B subunits of type II heat-labile enterotoxin would induce cellular immune response in a way similar to that induced by the A2 and B subunits of type I heat-labile enterotoxin.

In conclusion, the combined prior art references teach that the A2 and B subunits of type I heat labile enterotoxin are structurally different from that of type II heat labile enterotoxin, and these two types of enterotoxins possess similar activities in terms of toxicity, structural integrity and induction of humoral immune response (**Toida** et al., **Connell** et al., 1992, 1998). The lack of differentiation between type I and type II heat labile enterotoxins in **Rappuoli** et al. and **Schodel** et al. renders these

two references ambiguous and useless in providing any guidance. The combined prior art references, however, do not teach or suggest that the A2 and B subunits of type I and II heat-labile enterotoxins would have similar capacity in the induction of cellular immune response in spite of substantial structural differences. Hence, the cited prior art references do not provide one of ordinary skill in the art with the requisite reasonable expectation of successfully producing Applicant's claimed methods. Accordingly, the invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

The Examiner also contends that the instant situation is amenable to the type of analysis set forth in In re Kerkhoven, 205 USPQ 1069 (CCPA 1980) wherein the court held that it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose. Applicant respectfully disagrees. Applicant submits that the present invention is not amenable to the type of analysis set forth in In re Kerkhoven because, as discussed above, the prior art references do not teach or suggest two compositions (the A2 and B subunits of type I heat-labile enterotoxin vs. that of type II enterotoxin) each of which is useful for the same purpose (i.e. induction of cellular immune

response). Accordingly, Applicant submits that the invention as a whole is not *prima facie* obvious, and that the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) be reversed.

In the Advisory Action mailed August 26, 2003, the Examiner maintained the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) as being unpatentable over **Russell** et al. (US patent no. 6,030,624) in view of **Rappuoli** et al. (*Immunol. Today* 20:493, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 57:1347, 1989; *Vaccine* 8:569, 1990) and **Connell** et al. (*Immuol. Lett.* 62:117, 1998; *Infect. Immunity* 60:1653, 1992). Applicant respectfully requestes that the Board reverse this rejection.

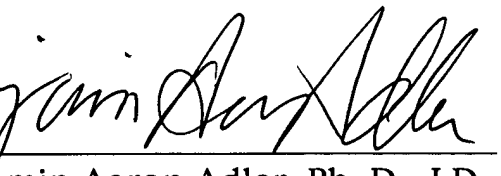
Russell et al. describe a method of inducing humoral (antibody) and cellular (T cell) immune responses using a chimeric immunogen comprising an antigen fused to the A2 and B subunits of cholera toxin (a type I heat-labile enterotoxin). The other cited references have been discussed above.

The Examiner rejects claims 1-3, 6 and 24-29 on the same basis as that based on **Toida** et al., **Rappuoli** et al., **Schodel** et al. and **Connell** et al. Therefore, the above discussion applies

here also. Applicant reiterates that the cited prior art references do not teach or suggest that the A2 and B subunits of type I and II heat-labile enterotoxins have similar capacity to induce cellular immune response in spite of substantial structural differences. The issue of whether the A2 and B subunits of type II heat labile enterotoxin can induce cellular immunity has to be determined by actual experimentation. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made. Accordingly, Applicant respectfully requests that the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) be reversed.

Respectfully submitted,

Date: Dec 5, 2003



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CLAIMS ON APPEAL

1. A method of inducing an immune response by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin, wherein said immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues, the development of cytotoxic T cells and immunological tolerance to the antigen sequence.

2. The method of claim 1, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

3. The method of claim 1, wherein said type II heat-labile enterotoxin is selected from the group consisting of *E. coli* heat-labile type IIa toxin and *E. coli* heat-labile type IIb toxin.

6. The method of claim 1, wherein said immunogen is administered by a route selected from the group consisting of

orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

24. A method of increasing Th1 response and cell-mediated immunity by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin.

25. The method of claim 24, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

26. The method of claim 24, wherein said immunogen is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

27. A method of increasing Th1 response and cell-mediated immunity by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a *E. coli* heat-labile type IIa or type IIb toxin.

28. The method of claim 27, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

29. The method of claim 27, wherein said immunogen is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

INFECTION AND IMMUNITY, Mar. 1997, p. 909–915
0019-9567/97/$04.00+0

Vol. 65, No. 3

Oral Immunization with the Saliva-Binding Region of *Streptococcus mutans* AgI/II Genetically Coupled to the Cholera Toxin B Subunit Elicits T-Helper-Cell Responses in Gut-Associated Lymphoid Tissues

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Received 15 May 1996/Returned for modification 17 July 1996/Accepted 30 November 1996

Mice immunized intragastrically (i.g.) with a genetically constructed chimeric protein consisting of the saliva-binding region (SBR) of *Streptococcus mutans* AgI/II coupled to cholera toxin (CT) A2 and B subunits (CTA2/B) develop serum immunoglobulin G (IgG) and mucosal IgA antibody responses against AgI/II that are enhanced by the coadministration of CT as an adjuvant. To investigate the development of antigen-specific T cells in the gut-associated lymphoid tissues, mice were immunized i.g. with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT. AgI/II-specific T cells in Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen were assayed by lymphoproliferation and flow cytometry for the expression of T-cell surface markers, and cytokine mRNA expression was evaluated by reverse transcription-PCR. T-cell responses were consistent with antibody responses but were detectable after the first immunization. Proliferative responses of PP and MLN cells upon stimulation with AgI/II in vitro were low and delayed in mice given SBR alone, and these cells displayed a mixed type 1 and 2 (or Th0) pattern of cytokine expression. Immunization with SBR-CTA2/B resulted in greater AgI/II-specific proliferative responses in PP cells and an increase in the proportion of $CD4^+$ T cells. Coadministration of CT with SBR-CTA2/B led to greater proliferative responses especially in the MLN cells, which then showed an increase in $CD4^+$ cells. Immunization with SBR-CTA2/B (with or without CT) skewed the cytokine expression pattern in PP and MLN cells toward Th2. The results indicate that T helper cells were induced in gut-associated lymphoid tissues by i.g. immunization with SBR-CTA2/B, concomitantly with and prior to the appearance of circulating and mucosal antibodies.

Initial adherence of *Streptococcus mutans* to tooth surfaces appears to be mediated largely by the 167-kDa surface fibrillar adhesin known as AgI/II (synonyms, antigen B, P1, SpaP, and PAc) (14). The adhesion domain that interacts with salivary pellicle has been located to the alanine-rich (A) repeat region in the N-terminal part of the molecule (2, 12) extending from the cell surface probably in an α-helical conformation (21). Early studies on AgI/II indicated that rhesus monkeys immunized with *S. mutans* and showing protection against dental caries mounted antibody responses especially against the complete molecule rather than against AgII (34), which corresponds to the C-terminal one-third. These results were supported by the finding that immunization with either complete AgI/II or the isolated AgI component (corresponding to the N-terminal two-thirds) afforded protection against caries (22). Thus, a rational approach to immunization against *S. mutans*-induced dental caries can be based on the generation of an appropriate antibody response in the saliva that would inhibit the adherence of *S. mutans* to tooth surfaces. Human secretory immunoglobulin A (S-IgA) antibodies to AgI/II have been shown to inhibit such adherence (14). However, S-IgA antibodies in saliva and other secretions are not effectively induced by conventional parenteral immunization (27).

S-IgA antibodies are most effectively induced by stimulating the common mucosal immune system (27), for example, by enteric immunization which stimulates the gut-associated lymphoid tissues, including the Peyer's patches (PP) of the small intestine. Considerable attention has been given to the development of improved procedures for the oral delivery of vaccines (28), one of which is coupling antigens to the nontoxic binding B subunit of cholera toxin (CT), a safe and highly immunogenic protein in humans (16). The B subunit of CT (CTB), because of its avid binding to G_{M1} ganglioside, which is present on all nucleated cell surfaces, is readily taken up by the M cells covering PP and passed to the underlying immunocompetent cells which initiate the mucosal IgA antibody response. Antigen-stimulated IgA-committed B cells, and corresponding T helper cells, then emigrate via draining lymphatics to the mesenteric lymph nodes (MLN) and thence via the thoracic duct to the circulation before relocating in the effector sites of mucosal immunity, such as the salivary glands. Here terminal differentiation of B cells into IgA-secreting plasma cells occurs, and their product, polymeric IgA, is transported through the glandular epithelium to form S-IgA. Several studies have shown that other antigens can be coupled to CTB to generate strong mucosal IgA antibody responses to the desired antigen (4, 26, 36) and that intact CT, though toxic, serves as an adjuvant that enhances the response to coadministered antigens (9, 23).

For immunization against *S. mutans*-induced caries, this laboratory has developed a potent oral immunogen consisting initially of AgI/II chemically coupled to CTB (20, 36, 43). Mice and monkeys immunized intragastrically (i.g.) or intranasally with this develop salivary IgA antibodies to AgI/II (35, 36, 43). Recently, a novel class of genetically engineered mucosal im-

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munogen, in which the 42-kDa saliva-binding region (SBR) of AgI/II is inserted in CT in place of the toxic CT A1 subunit to create a chimeric protein in which SBR is coupled to pentameric CTB by the CTA2 peptide, has been developed (11). This protein has been shown to induce a persistent salivary IgA antibody response against AgI/II when administered i.g.; CT coadministered as an adjuvant enhances the response. However, optimal responses to either the chemically conjugated AgI/II-CTB or the genetically constructed SBR-CTA2/B have appeared to require three doses given at 10-day intervals (11, 36). This finding implies that the first dose must prime the mucosal immune system to respond to second and subsequent doses of the immunogen. Such priming would be expected to elicit antigen-specific T helper cells in the PP and MLN. Furthermore, the enhancement of antibody responses by the adjuvant effect of CT might be expected to involve increased T-helper-cell activity. This study was undertaken to test these hypotheses by examining AgI/II-responsive T cells in the PP and MLN of mice during the course of i.g. immunization with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant.

MATERIALS AND METHODS

Antigens. AgI/II was purified chromatographically from the culture supernatant of *S. mutans* essentially as described previously (33).

The SBR-CTA2/B chimeric protein was constructed and expressed in *Escherichia coli* and purified from extracts as described previously (11). In essence, this procedure consisted of PCR amplifying DNA for a 42-kDa segment encompassing the A repeat region and some downstream sequence of AgI/II from the *pac* gene, ligating this in a modified pET20b(+) plasmid (Novagen, Inc., Madison, Wis.) in frame with and upstream of the genes for CTA2 and CTB, and transforming the recombinant plasmid into *E. coli* BL21(DE3) cells (Novagen).

SBR polypeptide was obtained by excising the relevant DNA and religating it into unmodified pET20b(+) in order to express SBR with a six-residue histidine sequence derived from the plasmid. This plasmid was also expressed in *E. coli* BL21(DE3), and SBR was purified from cell lysates by metal chelation chromatography on a nickel-loaded column (Novagen) according to the manufacturer's instructions.

CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell, Calif.).

Animals and immunization. Adult BALB/c mice of either sex, 14 to 20 weeks old, from a pathogen-free colony were used for all experiments. Groups of nine mice were immunized i.g. three times at 10-day intervals by gastric intubation of either SBR-CTA2/B (100 μg) alone, SBR-CTA2/B together with 5 μg of CT as an adjuvant, or an equimolar amount of SBR (40 μg), all given in 0.5 ml of 0.35 M $NaHCO_3$. Serum and saliva samples were collected on day 0 and 10 days after each immunization for assay of antibodies by enzyme-linked immunosorbent assay (ELISA). In some experiments, subgroups of three mice were killed 10 days after each immunization for the preparation of cells from PP, MLN, and spleens for T-cell proliferation and flow cytometric analyses.

ELISA. Serum IgG and salivary IgA antibodies to AgI/II and total salivary IgA concentrations were determined by ELISA as described previously (36) on plates coated with AgI/II and anti-mouse IgA, respectively, and by using goat anti-mouse IgG- and IgA-peroxidase conjugates (Southern Biotechnology Associates, Inc., Birmingham, Ala.) as detection reagents. Unknowns were interpolated on calibration curves constructed by a computer program based on four-parameter logistic algorithms as previously described (36).

Preparation and culture of lymphoid cells. Single-cell suspensions were obtained by teasing PP, MLN, and spleen apart with needles, and tissue debris was removed by filtering through nylon mesh. Peripheral blood mononuclear cells were obtained by centrifugation on Histopaque 1083 (Sigma Diagnostic, St. Louis, Mo.). Remaining erythrocytes were lysed in buffered ammonium chloride; the cells were washed three times in RPMI 1640 medium (Mediatech, Washington, D.C.) supplemented with 2% fetal calf serum (FCS) and were finally resuspended in 10% FCS–RPMI 1640. Cells were cultured in 10% FCS–RPMI 1640 supplemented with 1 mM sodium pyruvate, nonessential amino acids, 2 mM glutamine, 100 U of penicillin-streptomycin per ml, 25 mM HEPES, and 0.01 mM 2-mercaptoethanol.

Flow cytometry. Cell marker expression on freshly isolated cells was determined by double staining with biotinylated anti-CD4 (GK1.5) followed by avidin-phycoerythrin and either fluorescein isothiocyanate-conjugated anti-CD3 (145-2C11) or fluorescein isothiocyanate-conjugated anti-CD8 (53-6.72) and by incubation for 30 min at 4°C in 2% FCS–Dulbecco's phosphate-buffered saline with 0.02% NaN_3. Cells were washed, fixed in 1% paraformaldehyde overnight, and analyzed on a FACStar IV flow cytometer (Becton Dickinson, Mountain View, Calif.).

Proliferation assay. Cells from PP, MLN, and spleens were incubated at 10^5 cells/well (0.1 ml) in triplicate with a previously optimized concentration of AgI/II (0.5 μg/ml) for 5 days and were pulsed with [^{3}H]thymidine (0.5 μCi/well) 8 h before harvesting. Uptake of ^{3}H was counted by a liquid scintillation counter. The stimulation index was calculated as cpm (wells with AgI/II)/mean cpm (control wells).

Cytokine expression. The expression of cytokines by PP, MLN, and spleen cells after culture in vitro with or without AgI/II (0.1 μg/ml) for 24 h was determined by a reverse transcription (RT)-PCR procedure for the amplification of cytokine mRNA. Cells (5×10^6 to 7×10^6) were harvested from the cultures, washed thoroughly, and then lysed in 350 μl of lysing buffer for isolation of RNA, using an RNeasy kit (Qiagen Inc., Chatsworth, Calif.). RNA was redissolved in 40 μl of diethyl pyrocarbonate-treated water, and 2-μl samples were added to 18 μl of RT mixture (Perkin-Elmer, Foster City, Calif.) containing 1× PCR buffer, 5 mM $MgCl_2$, 1 mM (each) deoxyribonucleoside triphosphate, 1 U of RNase inhibitor per ml, 2.5 U of Moloney murine leukemia virus reverse transcriptase per ml, and 2.5 mM oligo$(dT)_{16}$. Mixtures were overlaid with 50 μl of light mineral oil and incubated in a thermal cycler (Perkin-Elmer) for 15 min at 42°C, 45 min at 37°C, 5 min at 99°C, and 5 min at 4°C. After RT 80 μl of PCR mixture (Perkin-Elmer) was added to each tube to give final concentrations of 25 U of AmpliTaq DNA polymerase per ml, 0.15 μM 5′ primer, 0.15 μM 3′ primer, 2 mM $MgCl_2$, and 1× PCR buffer II. Primers specific for murine gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, and β-actin (30) were obtained from Clontech Laboratories Inc. (Palo Alto, Calif.) or the Oligonucleotide Synthesis Core Facility of the UAB Comprehensive Cancer Center, and their specificities were verified by means of RT-PCR on RNA extracted from mitogen-stimulated mouse spleen cells. After heating at 95°C for 2 min, cDNA was amplified for 35 cycles consisting of 45 s at 94°C, 3 min at 72°C, and 2 min at 60°C. The products of amplification were analyzed by 2% agarose gel electrophoresis, revealed by ethidium bromide staining, and photographed by UV transillumination. The results were scored according to the presence or absence of a band of appropriate molecular size.

Statistical methods. Quantitative results were evaluated by Student's *t* test, by means of MultiStat (Biosoft, Ferguson, Mo.) on a Macintosh computer. Antibody data were transformed logarithmically to normalize their distribution and homogenize the variances.

RESULTS

Antibody responses. Intragastric immunization of mice with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT incrementally induced serum IgG and salivary IgA antibodies measured against whole AgI/II (Fig. 1). Immunization with SBR alone resulted in weak but statistically significant ($P < 0.001$ at all intervals) serum IgG antibody responses and modest salivary IgA antibodies that were significantly elevated above the background level only after the second and third immunizations ($P < 0.001$ and $P < 0.01$, respectively). Administration of the SBR-CTA2/B chimeric protein generated significantly greater serum IgG responses ($P < 0.001$), and coadministration of CT as an adjuvant further enhanced both the level and the earlier development of serum IgG antibodies. Salivary IgA antibodies also tended to be elevated by immunization with SBR-CTA2/B chimeric protein, especially when given with CT as an adjuvant; however, because of variation between animals, statistical significance was attained only after two doses given with CT. Nevertheless, the general pattern of results was in accordance with expectations based on responses to AgI/II, either alone or chemically conjugated to CTB, administered i.g. without or with CT adjuvant (4, 36). Total salivary IgA concentrations also increased in all animals during the immunization period, from 2.13 ± 0.61 μg/ml in unimmunized animals to 5.92 ± 0.64 μg/ml after three immunizations, but there were no significant differences between the immunization groups.

T-cell proliferative responses. To test whether T cells capable of proliferating in vitro in response to stimulation with AgI/II had been induced by the first, second, or third i.g. dose, groups of three mice were killed 10 days after a first, second, or third immunization with each immunogen preparation. This time interval corresponded to the immunization interval and was chosen to permit assessment of the potential responsiveness of cells sensitized by the previous immunization to the next dose. Mononuclear cells from PP, MLN, and spleens were

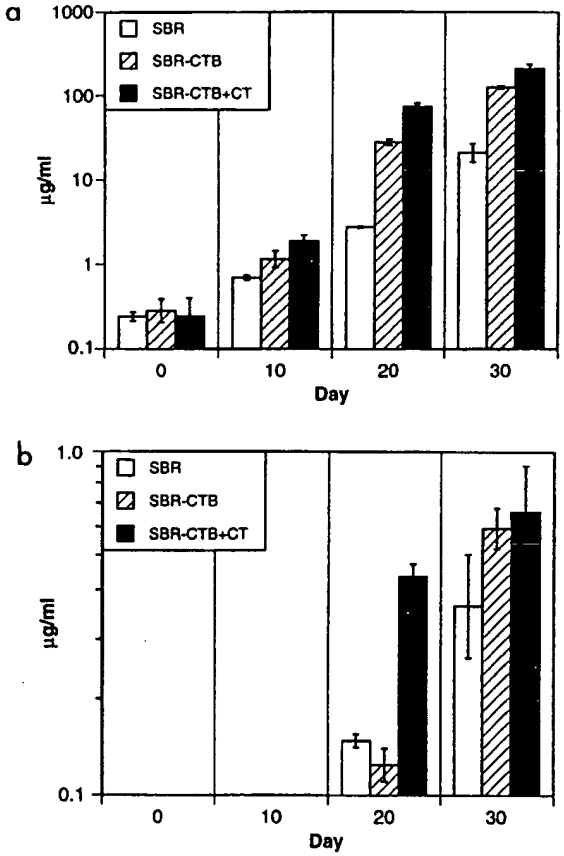

FIG. 1. Serum IgG (a) and salivary IgA (b) antibody responses to AgI/II in unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Immunizations were given on days 0, 10, and 20, and samples were collected 10 days after each immunization, i.e., on days 0 (unimmunized mice), 10 (one dose), 20 (2 doses), and 30 (3 doses). Results shown are the geometric mean and standard deviation, plotted on logarithmic ordinates, of samples from three animals analyzed separately. Salivary IgA antibodies were below detectable levels (<0.1 μg/ml) on days 0 and 10.

cultured with or without AgI/II. Incorporation of [^{3}H]thymidine expressed as stimulation indices revealed that AgI/II-responsive cells were elicited in the lymphoid tissues associated with the intestine, incrementally with the number and form of the immunogen doses (Fig. 2). PP and MLN cells taken from mice given two or three doses of SBR or of SBR-CTA2/B alone showed modest proliferative responses to AgI/II in vitro (stimulation indices in the range of 2.4 to 3.2; 5.44 for PP from mice given three doses of SBR-CTA2/B), whereas PP and MLN cells from mice immunized with SBR-CTA2/B plus CT adjuvant showed proliferative responses after one dose (stimulation indices of 2.3 and 3.6, respectively) and greater responses after two or three doses (stimulation indices of 3.1 to 6.1). The proliferative responses of PP and MLN cells were different: MLN cells responded similarly to (or less than) PP cells when taken from mice immunized with SBR or SBR-CTA2/B but showed greater responses to AgI/II in vitro when taken from mice given AgI/II-CTA2/B plus CT. Spleen cells generally did not respond to stimulation with AgI/II in vitro (stimulation indices of <2), except for those taken from mice immunized once with SBR-CTA2/B plus CT (stimulation index of 2.8). Cells from the PP, MLN, or spleens of unimmunized mice did not proliferate in response to AgI/II in vitro (stimulation indices of 1.2 to 1.5).

T-cell surface marker analysis. To elucidate the nature of the T-cell responses to i.g. immunization, cells freshly isolated from PP, MLN, spleen, or peripheral blood of mice immunized once, twice, or three times with the different immunogens were analyzed by flow cytometry for the proportion of cells expressing T-cell marker CD3 (all T cells), CD4 (T helper phenotype), or CD8 (T suppressor/cytotoxic phenotype). The results are shown in Fig. 3. Among PP cells, there was an increase in the proportion of total T cells after each immunization that was most noticeable in animals immunized with SBR-CTA2/B or SBR-CTA2/B plus CT; this increase was mostly in the CD4$^+$ T helper population, whereas the CD8$^+$ T suppressor/cytotoxic population remained small. The MLN cell populations remained more stable, except in the case of cells from mice immunized with SBR-CTA2/B plus CT, in which the CD4$^+$ population increased with the number of immunizations. MLN generally, however, contained more T cells of both phenotypes than PP, regardless of immunization status. Peripheral blood cells tended to show the greatest increases in the proportion of CD4$^+$ T cells after immunization, especially with SBR-CTA2/B plus CT, although these numbers must be interpreted with caution because of the small numbers of cells obtained. Spleen cells showed modest increases in the proportions of CD4$^+$ T cells after immunization in all groups.

Cytokine expression. To elucidate the pattern of expression of cytokines, PP, MLN, and spleen cells were taken from mice immunized three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT 3 days after the last dose, cultured in vitro for 24 h with or without AgI/II, and examined for the presence of mRNAs for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and β-actin by RT-PCR. This time interval was chosen because previous experience indicated that ex vivo analysis of cytokine production

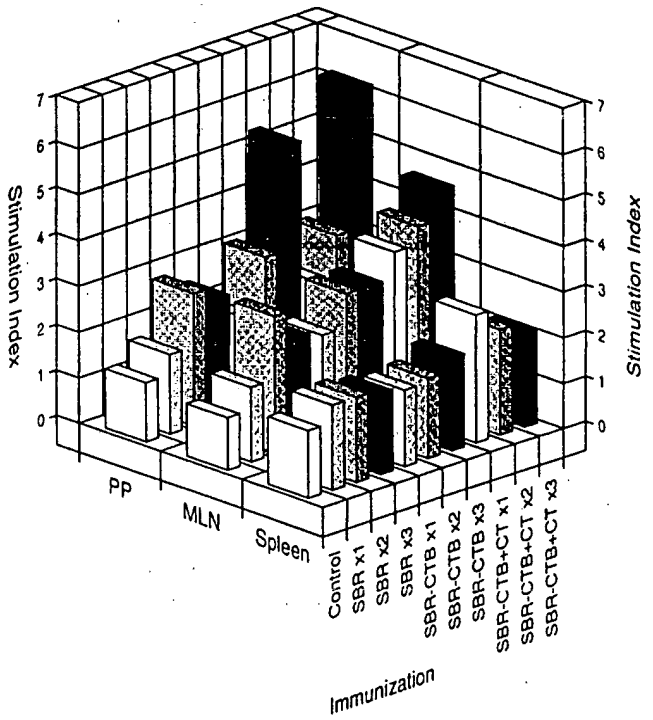

FIG. 2. Proliferative responses of cells from PP, MLN, and spleens of unimmunized (control) mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant, cultured in vitro with AgI/II. Results shown are mean stimulation indices of three replicate cultures; standard deviations ranged from ±0.04 to ±0.95.

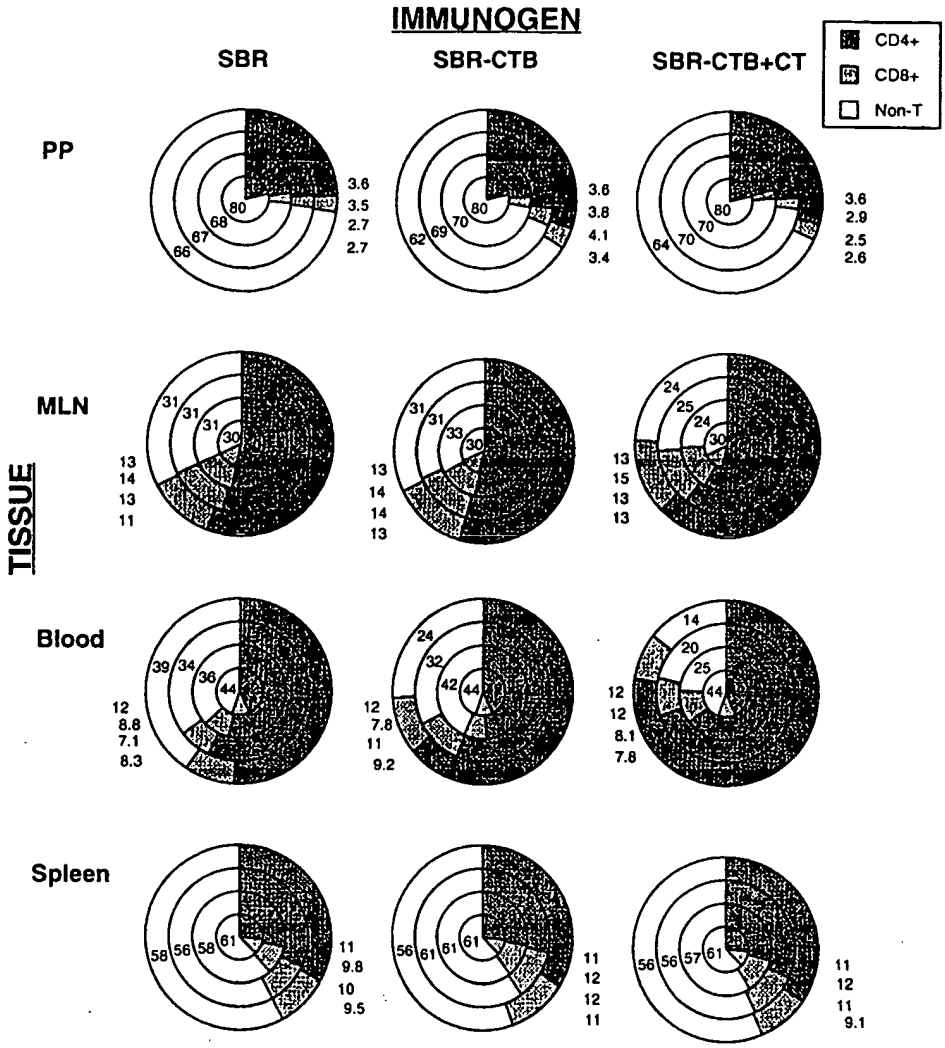

FIG. 3. Phenotypic analysis of cells from PP, MLN, peripheral blood, and spleens of unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Each pie shows the proportions of $CD4^+$, $CD8^+$, and $CD3^-$ (non-T) cells as a percentage of total gated mononuclear cells determined by flow cytometry, starting with unimmunized mice (center of each pie) and proceeding outward in concentric rings with mice immunized once, twice, or three times. Numbers within the rings are the individual percentages of each phenotype of cells (for clarity, $CD8^+$ cell data are shown outside the pies in descending order: zero, one, two, and three doses); the value shown for MLN from mice immunized once with SBR (marked as 51?) was not obtained experimentally but was inserted for plotting purposes as the average of the values either side of it. Note that the numbers in each ring of a pie do not sum to exactly 100% because of the presence of some $CD4^-$ $CD8^-$ (double-negative) $CD3^+$ T cells and possibly some $CD4^+$ $CD8^+$ (double-positive) T cells in each cell preparation.

was best assessed within a few days after immunization. All cultures generated β-actin PCR products of similar band intensities. After culture with AgI/II, PP, MLN, and spleen cells from mice immunized with SBR alone revealed mRNAs for IFN-γ and IL-2, but only PP and spleen cells also revealed IL-4 mRNA, whereas IL-5 mRNA was detectable in all cell cultures regardless of stimulation (Table 1). PP cells from mice immunized with SBR-CTA2/B, without or with CT adjuvant, did not reveal mRNA for IFN-γ or IL-2, even after culture with AgI/II, and MLN cells from these animals revealed variable IFN-γ and IL-2 mRNA responses. However, PP, MLN, and spleen cells revealed IL-4 mRNA particularly after stimulation with AgI/II, whereas all cultures were positive for IL-5 mRNA. Likewise, mRNAs for IL-6 and IL-10 were found in all cell cultures, regardless of immunization or in vitro stimulation (data not shown). Most notably, immunization with SBR-CTA2/B (without or with CT) resulted in a decrease of AgI/II-specific Th1 activity, as revealed by diminished expression of IFN-γ and IL-2 mRNAs in PP cells, and increased Th2 activity (IL-4 mRNA expression) in MLN cells in comparison with immunization with SBR alone (Table 1). There was an increase in IFN-γ and IL-2 expression (in response to stimulation with AgI/II in vitro) in PP, MLN, and spleen cells from mice immunized three times with SBR alone relative to cells from mice immunized twice (not shown). Likewise, spleen cells from mice immunized three times with SBR-CTA2/B (without or with CT) showed increased AgI/II-specific expression of IFN-γ, IL-2, and IL-4 relative to twice-immunized mice. Cells from unimmunized mice did not respond in culture with AgI/II by the expression of IFN-γ, IL-2, and IL-4 mRNAs above that revealed in control cultures, except that spleen cells showed weak evidence of IFN-γ expression upon culture with AgI/II. Thus, PP and MLN cells from mice immunized with SBR alone revealed type 1 (IFN-γ and IL-2) as well as type 2 (IL-4) cytokine responses upon stimulation in vitro, whereas cells from the same organs of mice immunized with SBR-CTA2/B

TABLE 1. Cytokine expression in PP, MLN, and spleen cell cultures of mice immunized with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT

Immunization[a]	Culture[b]	mRNA expression[c]											
		IFN-γ			IL-2			IL-4			IL-5		
		PP	MLN	Spleen	PP	MLN	Spleen	PP	MLN	Spleen	PP	MLN	Spleen
SBR	Control	−	−	−	−	−	−	−	−	−	+	+	+
	+AgI/II	+	+	+	+	+	+	+	−	+	+	+	+
SBR-CTA2/B	Control	−	−	−	−	−	−	+	−	+	+	+	+
	+AgI/II	−	−	+	−	+	+	+	+	+	+	+	+
SBR-CTA2/B + CT	Control	−	−	+	−	−	+	−	−	+	+	+	+
	+AgI/II	−	+	+	−	−	+	+	+	+	+	+	+

[a] Mice were immunized three times at 10-day intervals, and organs were collected 3 days after the last immunization.
[b] Cells were cultured in vitro for 24 h without (control) or with AgI/II (0.1 μg/ml).
[c] Cytokine mRNA expression detected by RT-PCR and scored according to the presence of an ethidium bromide-stained band of appropriate molecular size. −, no detectable band; +, clear band.

chimeric protein (without or with CT) revealed IL-4 but little or no type 1 cytokine response.

DISCUSSION

Lymphocytes taken from the PP and MLN of mice immunized i.g. with SBR or with SBR-CTA2/B without or with CT as adjuvant were capable of proliferating in vitro when stimulated with AgI/II, showing similar overall patterns of T-cell responses to the different regimens and stages of immunization as the serum and salivary antibody responses. Immunization with SBR alone induced the lowest proliferative responses in PP and MLN cells, and this was reflected also in the finding that there was little change in the proportions of $CD4^+$ and $CD8^+$ T cells in these organs. Moreover, the pattern of cytokine expression in the cells from PP and MLN of these mice suggested a mixed type 1 and type 2 helper activity, possibly arising from the different types of cells in the culture, or indicating regulation by Th0 cells (8). Coupling SBR to CTB in the form of the SBR-CTA2/B chimeric protein enhanced its immunogenicity with respect to T-cell responses in PP and MLN, and the addition of CT as an adjuvant further elevated these responses. Furthermore, the cytokine expression pattern in PP and MLN cells from mice immunized with SBR-CTA2/B (with or without CT) indicated that T-cell help was skewed toward Th2 activity. In part, these shifts might be explained by the enhanced migration of cells from PP to MLN and thence into the circulation and effector sites of mucosal immunity, but the decrease in Th1 cells in PP was not matched by a corresponding increase in MLN or spleen in this cross-sectional study. The finding of IL-5, IL-6, and IL-10 mRNAs in cell cultures regardless of antigen stimulation in vitro is not readily explained in terms of enhanced Th2 cell activity but may indicate constitutive expression of these cytokines or their continued expression ex vivo after immunization. It is also possible that IL-6 and IL-10 mRNAs were derived from macrophages present in the cell cultures, although these would be largely adherent and unlikely to be harvested along with the lymphocytes.

The proportions of $CD4^+$ T cells in PP increased after each additional dose of these immunogen preparations, but a corresponding increase was seen in MLN cells only from mice immunized with SBR-CTA2/B chimeric protein and CT adjuvant. The finding that these T-cell responses occurred in PP and MLN as early as after the first immunization, at least with SBR-CTA2/B, showed that antigen-sensitized T cells were elicited before IgA antibody responses became elevated in the effector sites of mucosal immunity such as salivary glands. The responses in MLN and PP were different, as significant proliferative responses and increased proportions of $CD4^+$ cells during the course of immunization were developed in MLN cells only when CT was used as an adjuvant, and moreover, MLN from all mice contained higher proportions of T cells of both phenotypes than corresponding PP. The proportion of $CD8^+$ cells was higher in MLN than in PP, but as it was not reduced by the administration of CT as an adjuvant, it appears that the enhanced AgI/II-specific proliferation in MLN cells from mice given CT is not due to inhibition of $CD8^+$ suppressor cells by CT (10). The spleen, a nonmucosal lymphoid organ, displayed little or no response in terms of antigen-specific proliferating T cells, despite the considerable elevation of serum IgG antibodies especially when SBR-CTA2/B was given together with CT adjuvant. This finding is consistent with the relatively modest numbers of specific antibody-secreting cells found in the spleen after i.g. immunization with AgI/II chemically conjugated to CTB and given with CT (36, 43). It is noteworthy that throughout these experiments, although the mice were immunized with SBR or SBR-CTA2/B chimeric protein, which represents residues 186 to 577 of AgI/II, both antibody and T-cell responses could be detected with intact AgI/II. This finding implies that SBR retains sufficient conformational structure similar to that of the corresponding part of the whole AgI/II molecule and that both are processed similarly by antigen-presenting cells.

These responses are in accordance with the concept of the common mucosal immune system and the dissemination of antigen-sensitized T and B cells from the inductive sites such as PP through the MLN that drain the lymph flow from the small intestine and thence into the circulation prior to relocation in the effector sites of mucosal immunity, including the salivary glands (27). Thus, i.g. immunization with SBR, especially when coupled to CTB in the form of a chimeric protein, leads to the appearance of antigen-responsive T cells in both PP and MLN. Because few cells were recoverable from blood, it was not practically possible to trace the appearance of such cells in the circulation, although this has been well documented in humans (1, 38), and the transient circulation of specific antibody-secreting cells, predominantly of the IgA isotype, approximately 1 week after mucosal immunization has been demonstrated in human and animal systems (3, 19, 35, 36). Curiously, perhaps, it appears that the peak of circulating antigen-specific T cells occurs after the peak of circulating antibody-secreting cells (1), and in the present experiments, an increased proportion of $CD4^+$ T helper cells was found in the peripheral blood of mice 10 days after the second or third dose of SBR-CTA2/B, especially if CT was also given as an adjuvant. Cytokine-secreting T cells are known to occur in effector sites of mucosal immunity,

such as the intestinal lamina propria and salivary glands (15, 32).

CT has been shown to enhance T helper responses in intestinal tissues, and particularly the response of the Th2 subset that is held to promote high levels of serum IgG and mucosal IgA antibody responses (17, 25, 29, 39, 44). We have likewise found evidence of type 2 cytokine production by antigen-specific T cells in nasal passage-associated lymphoid tissue and the draining cervical lymph nodes of mice immunized intranasally, as well as in PP and MLN of mice immunized i.g., with AgI/II conjugated to CTB (41, 42). CT has also been reported to deplete selectively $CD8^+$ intraepithelial lymphocytes (10), and while the functions and migratory potential of these cells are incompletely understood, any such effect within inductive sites such as the PP would also serve to elevate the proportion of $CD4^+$ T cells. However, in this study, although the proportion of $CD8^+$ cells declined slightly in some tissues, this decrease appeared to occur concomitantly with an increase in the number of $CD3^+$ cells, in particular the $CD4^+$ subset. Whether CTB itself can serve as an adjuvant in the absence of intact CT has been controversial. Synergism between CTB and CT has been demonstrated (23, 37, 40), and most commercially available, nonrecombinant preparations of CTB contain small amounts of intact CT that may be sufficient to show this effect. Working with chemical conjugates of AgI/II and CTB delivered i.g., we previously demonstrated that even with nonrecombinant CTB, it was necessary for the antigen to be coupled to CTB and for intact CT to be coadministered (4, 36). However, both this study and our previous reports (11, 13) show that the genetically constructed SBR-CTA2/B chimeric protein, in which the toxic CTA1 subunit has been deleted, is clearly able to induce mucosal and circulating antibodies without the necessity for additional CT. Although the adjuvant activity of CT may be closely linked to its toxicity, which is a function the ADP-ribosyltransferase activity of the A1 subunit (24), recent reports suggest that adjuvanticity of the related *E. coli* heat-labile enterotoxin can be dissociated from toxicity (6, 7). Fusion proteins of CTB directly coupled to other antigenic peptides have been constructed, but the conformation of CTB and its ability to form G_{M1}-binding pentamers tend to be disrupted by peptides longer than approximately 12 amino acid residues (5, 31), and moreover, their mucosal immunogenicity seems to be limited in the absence of additional CT. These limitations do not apply to SBR-CTA2/B chimeric protein, in which a large 42-kDa segment of protein is fused to the CTA2 subunit, which couples it noncovalently to the CTB pentamer to preserve its G_{M1} ganglioside-binding activity. The enhanced enteric immunogenicity of SBR-CTA2/B chimeric protein, even in the absence of CT, is advantageous for an oral vaccine, as recombinant CTB has been shown to be a safe and effective immunogen in humans (18).

We therefore conclude that i.g. immunization with SBR, especially when genetically coupled to CTB to enhance both mucosal and circulating antibody responses, induces T-cell responses in the gut-associated lymphoid tissues such as PP and MLN. Furthermore, these T-cell responses occur after one or two doses of immunogen, earlier than the antibody responses, and include increased proportions of $CD4^+$ T helper cells. The responses are enhanced by, but are not dependent on, the addition of CT as an adjuvant.

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Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins

Rino Rappuoli, Mariagrazia Pizza, Gill Douce and Gordon Dougan

Escherichia coli heat-labile enterotoxin and cholera toxin are potent mucosal immunogens and adjuvants in animal models. Non-toxic mutants retaining adjuvant activity are useful tools to dissect the mechanism of mucosal adjuvanticity and promising candidates for development of human vaccines and immunotherapy. Clinical trials are expected to proceed in the near future.

The mucosal surfaces of the body are constantly exposed to a myriad of benign foreign antigens that are acquired through eating, breathing and touching, among others. Superficially, environmental or food antigens appear to be substantially ignored by the healthy immune system, which regards them as harmless. Indeed, we might actually show measurable immunological tolerance to them[1]. Relatively few molecules are highly immunogenic when they contact mucosal surfaces, in the sense that they generate strong humoral and secretory antibody responses. Such molecules are often referred to as mucosal immunogens.

The most powerful mucosal immunogens that are recognized to date are cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT), the molecules that cause the debilitating watery secretions typical of cholera and traveller's diarrhoea, respectively[2,3]. The mucosal immune system somehow recognizes that these toxins are a threat and, a short time after they make contact with a mucosal surface, a powerful immune response is mounted against them. This antitoxin response is so potent that sometimes a strong and easily measurable immune response is also activated against foreign bystander molecules that are present simultaneously at the mucosal surface[4].

As a consequence of this immunopotentiating property, CT and LT have been investigated extensively and exploited as mucosal immunogens and adjuvants in animal models[5,6]. However, the high toxicity of CT and LT makes them unsuitable for practical human use[2], thus prompting recent efforts to dissect the mucosal immunogenicity and adjuvanticity of CT and LT from their toxicity. Site-directed mutagenesis, guided by the crystal structure of the molecule and coupled with molecular modelling[7], has clarified our understanding of what makes these molecules so special in terms of mucosal immunity by disclosing the role of the receptor-binding domain, the B subunit, the A subunit and the enzymatic activity of LT and CT (Ref. 8). Critically, the use of highly purified recombinant material has also clarified compromised observations made previously with toxin-contaminated LT and CT B subunit derivatives (LTB and CTB, respectively)[8–10].

The relationship between structure and function of LT and CT

CT and LT belong to the AB class of bacterial toxins[11]. The two molecules have high homology (80% identity) in their primary structure[12,13] and superimposable tertiary structures[14]. Both toxins are composed of a pentameric B oligomer that binds the receptor(s) on the surface of eukaryotic cells, and an enzymatically active A subunit that is responsible for the toxicity (Fig. 1).

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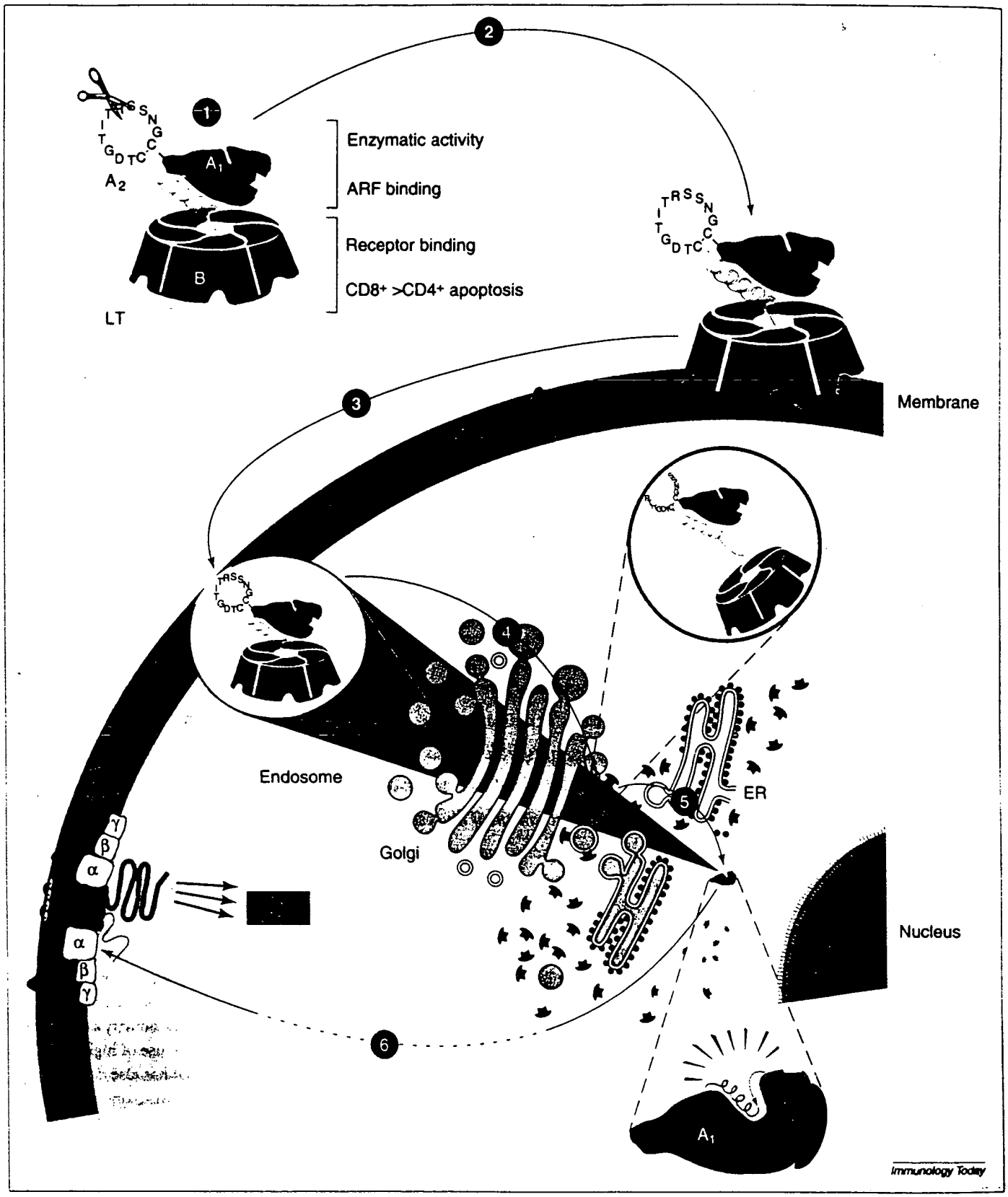
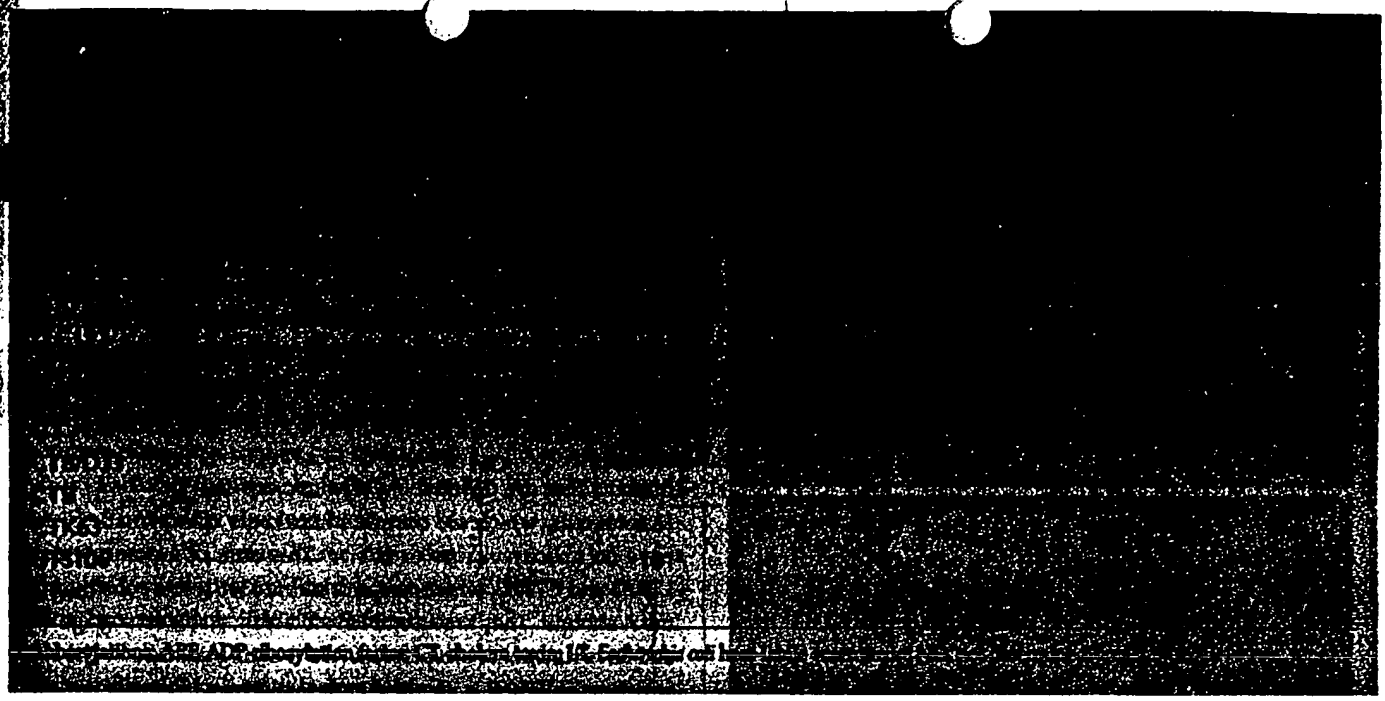


Fig. 1. *The sequence of events during cell intoxication. (1) Schematic representation of heat-labile enterotoxin (LT), showing the A and B subunits, their structural and functional significant features and the site of proteolytic cleavage of the loop between the A_1 and A_2 domains. (2) LT binds the receptor located on the plasma membrane of eukaryotic cells. (3) LT is internalized into vesicles. (4) The vesicles are transported to the Golgi apparatus where the holotoxin is disassembled. (5) The A subunit is transported from the Golgi to the endoplasmic reticulum (ER), the A or the A_1 subunit is translocated from the ER to the cytosol, where it can interact with the soluble ADP-ribosylation factor (ARF). (6) The activated A_1 migrates to the plasma membrane where the substrate G_s is located. The ADP-ribosylation of the α subunit of G_s induces permanent activation of adenylate cyclase and intracellular accumulation of cAMP. Abbreviations: Adc, adenylate cyclase; cAMP, cyclic AMP.*



The B oligomer

The B oligomer is a pentameric molecule of 55 kDa, containing five identical polypeptide monomers. The structure is compact, trypsin-resistant and requires boiling in the presence of sodium dodecyl sulphate to be dissociated. The five subunits are arranged in a cylinder-like structure, with a central cavity that exposes, on one side, five symmetrical cavities that are responsible for binding to the eukaryotic cell receptor[14] (Fig. 1). The receptor binding site is specific for a variety of galactose-containing molecules and shows a different fine specificity between LT and CT. CT binds mostly to the ganglioside GM1, which is believed to be the major toxin receptor[15], whereas LT binds not only GM1 (Ref. 16) but also other glycosphingolipids[17], glycoprotein receptors present in the intestine of rabbits and humans[18,19], polyglycosilceramides (PCGs)[20] and paragloboside[17]. Furthermore, the two variants of LT, human LT (hLT) and porcine LT (pLT), which differ by only four amino acids[21], are identical in their binding to glycoproteins and PCGs, but different in binding to paragloboside; pLT but not hLT binds paragloboside[20]. The different receptor binding activities of the LT and CT might be significant for the qualitatively different immunological properties that are exhibited by the two molecules, as will be discussed later.

Overall, two properties are associated with the B oligomer: (1) the ability to bind the receptor; and (2) to induce apoptosis of $CD8^+$ cells and, to a lesser extent, $CD4^+$ T cells (Fig. 1).

The A subunit

The A subunit is composed of a globular structure linked to the B oligomer by a trypsin-sensitive loop and a long α helix, the C-terminus of which enters into the central cavity of the B oligomer, thus anchoring the A subunit to the B pentamer[14]. Following protease cleavage of the loop, the A subunit is divided into the globular (enzymatically active) A_1 and the C-terminal A_2 fragments that remain linked by a disulphide bridge between the A_1–Cys187 and the A_2–Cys199 (see Fig. 1). Proteolytic cleavage of the loop and reduction of the disulphide bridge are both necessary for activation of the enzyme[22]. This loop is uncleaved when the molecules are produced in *E. coli*, however it is cleaved by a specific protease when molecules are produced in *Vibrio cholerae*[23]. The A_1 subunit contains an ADP-ribosylating enzymatically active pocket that binds nicotinamide adenine dinucleotide (NAD) and transfers the ADP-ribose group to the α subunit of several GTP-binding proteins that are involved in signal transduction. The consequences of transferral to G_s, the GTP-binding protein that regulates the activity of adenylate cyclase, are the best studied[11,24,25]. G_s ADP-ribosylation causes permanent activation of adenylate cyclase and abnormal intracellular accumulation of cAMP (Ref. 26; Fig. 1).

A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa GTP-binding proteins, known as ADP-ribosylation factors (ARFs). ARFs play a crucial role in vesicular membrane trafficking in both endocytic and exocytic pathways, and contribute to the maintenance of organelle integrity and assembly of coat proteins in eukaryotic cells[27]. Overall, the A_1 fragment can be seen as having at least two independent functions: enzymatic activity and ARF binding (Fig. 1).

The toxic sequence

The sequence of events that takes place during intoxication of eukaryotic cells[28–30] can be summarized as follows (see Fig. 1). The toxin binds the receptor and is internalized into vesicles that transport it to the Golgi compartment. Subsequently, the A and B subunits are dissociated, and the A subunit is transported from the Golgi to the endoplasmic reticulum (ER), whereas the B subunit persists in the Golgi and is later degraded. The A_1 subunit is then translocated from the ER to the cytosol, where it can interact with the soluble ARF and be activated. Finally, the A_1 subunit ADP-ribosylates the α subunit of G_s and possibly other G proteins located on the plasma membrane.

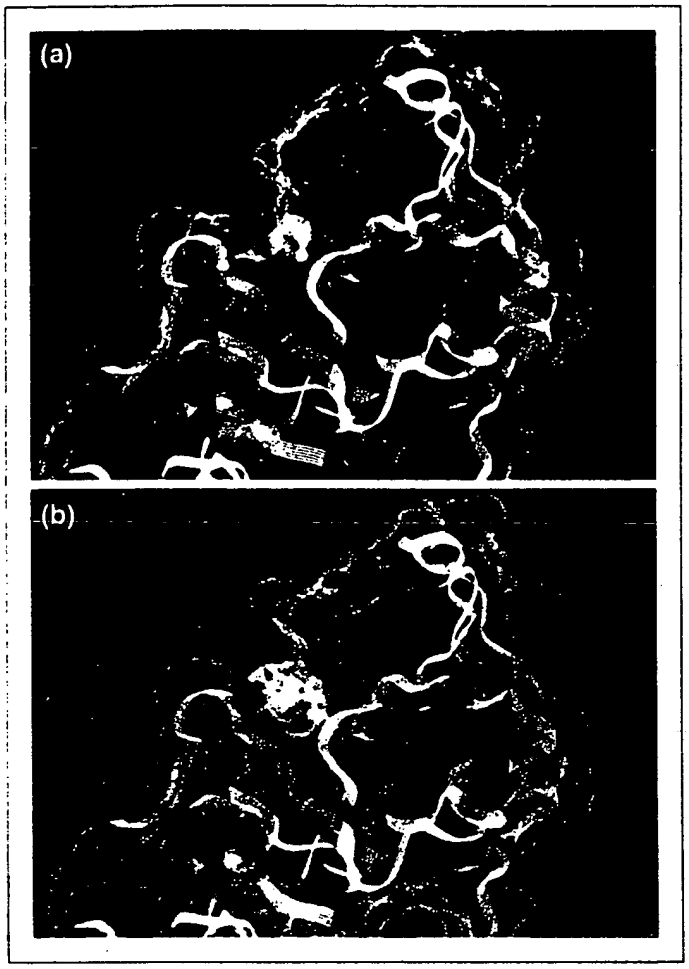


Fig. 2. *Three-dimensional structure of the A subunits of (a) wild-type heat-labile enterotoxin (LT) and (b) LTK63 represented as α-carbon trace and as solvent-exposed surfaces. The α helix and the β strand that form the nicotinamide adenine dinucleotide (NAD)-binding site are highlighted in red; the residues at position 63 [serine in the wild-type LT (a) and lysine in the LTK63 mutant (b)] are shown in yellow. The large, charged side-chain of the lysine in position 63 fills the NAD-binding cavity, thus obstructing it.*

Mutant toxin production and evaluation

To study the structure–function of CT and LT as well as to define molecules that are non-toxic but still active as mucosal adjuvants and immunogens, more than 50 different site-directed mutants have been produced[8–10,31–39]. The best characterized and most relevant are described below (see Table 1).

Mutants of the B subunit

Independently expressed B subunits were the first non-toxic derivatives of CT and LT to be produced. An oral vaccine against cholera, which contains recombinant CTB as a component, has been developed and thoroughly evaluated in animals and humans[40]. In this vaccine, CTB seems to act only as an immunogen and not as an adjuvant. Initial commercial preparations of CTB, in which the B subunit was purified from the active toxin, were associated with adjuvant activity; however, much of this activity was derived from contamination with active CT (Refs 41, 42). LTB seems to have a small variable but detectable adjuvant effect[8,10,43].

A mutant in LTB, defective in receptor binding (LTB/D33, containing a glycine to aspartic acid substitution at position 33), is non-immunogenic at mucosal surfaces, suggesting that an intact receptor binding site is necessary both for binding and immunogenicity[44]. Whether LT mutants that are defective in receptor binding, with and without enzymatic activity, are still active as adjuvants is controversial[37,45]. Non-binding LTB mutants also lose other immune-modulating activities, including their ability to induce apoptosis of $CD4^+$ and $CD8^+$ cells[44,46].

Mutants that are deficient in enzymatic activity

Holotoxoids, which are complete knockouts of enzymatic activity, have no toxicity *in vitro* or *in vivo*. This class of mutants includes LTK63 and CTK63, which contain a serine 63 to lysine substitution in the A subunit. They are assembled efficiently, stable on storage[32–34,47] and have functional receptor and ARF-binding domains[48]. The X-ray structure of LTK63 is identical to the wild-type LT across the entire molecule, with the exception of the active site where (as shown in Fig. 2) the bulky side chain of lysine 63 fills the catalytic cavity, thus making it unsuitable for enzymatic activity[49]. LTK63 is an excellent mucosal adjuvant, although the activity is reproducibly reduced in comparison with LT (Refs 8, 10, 50–53; Table 1; Fig. 3a), whereas CTK63 is a poor adjuvant[10] (Table 1; Fig. 3a). This adjuvant activity has been demonstrated using a wide range of antigens, including model antigens such as ovalbumin and protective antigens from bacterial and viral pathogens. Interestingly, LTK63 is consistently a better immunogen than LTB (Refs 8, 33, 39), suggesting an important role for the enzymatically inactive A subunit in the induction of an immune response. This is a property that might reflect not only the larger number of B- and T-cell epitopes provided by the A subunit but also its ability to influence intracellular events, such as antigen processing and presentation. In addition, the poor adjuvanticity of CTK63 has been associated with poor immunogenicity[10]. Differences between the adjuvant activity of LTK63 and CTK63 might reflect the effect of the different receptor binding affinities of these proteins. Other CT holotoxoid mutants that are described in the literature as mucosal adjuvants include CTF61 and CTK112 (containing serine 61 to phenylalanine and a glutamic acid 112 to lysine substitutions, respectively). However, it is important to point out that in those experiments, the amount of CT mutants used was ten times higher than that of wild-type CT (Ref. 38). These controversial results will be discussed later.

A further class of mutant molecules contains LTR72 (with an alanine to arginine substitution in position 72 of the A subunit) and CTS106 (with a proline to serine substitution in position 106 of the A subunit). These mutants have approximately 1% of the wild-type ADP-ribosylating activity, *in vitro* toxicity in Y1 cells reduced by a factor of 10^4–10^5 and approximately 1% toxicity *in vivo* (Fig. 3b). Both LTR72 and CTS106 are excellent mucosal adjuvants, being as effective as LT and CT, respectively[8,10] (Table 1; Fig. 3a). These two mutant holotoxoids might have their toxicity reduced sufficiently for safe use in humans, although still maintaining a little enzymatic activity, which significantly enhances their adjuvanticity.

Mutants in the protease-sensitive loop

Mutants in this region were constructed to make the loop insensitive to proteases and hence eliminate the susceptibility of the toxin to the cleavage required for activation of the enzymatic activity and toxicity. The best characterized mutant is LTG192, in which arginine 192 is replaced by a glycine[35,54,55]. *In vitro*, the mutant is completely trypsin-resistant; however, *in vivo*, proteases other than trypsin can cleave the loop and activate the toxin, as toxicity is detectable. The toxicity observed in Y1 cells is approximately 10^3 times lower than the wild-type toxin during the first 8 h of incubation, becoming only 5–10 times lower than wild-type following longer incubation[54]. In practice, this molecule takes longer to be activated but delivers approximately the same total enzymatic activity as wild-type. The difference is that the delivery of the active toxin is diluted over a longer period of time. *In vivo*, in the rabbit ileal loop, very little difference in toxicity is observed between LTG192 and wild-type LT (Ref. 56; Fig. 3b). Ongoing human trials are expected to establish the safety profile of this molecule[56]. LTG192 is indistinguishable from wild-type toxin both in terms of immunogenicity and adjuvanticity (Table 1; Fig. 3a).

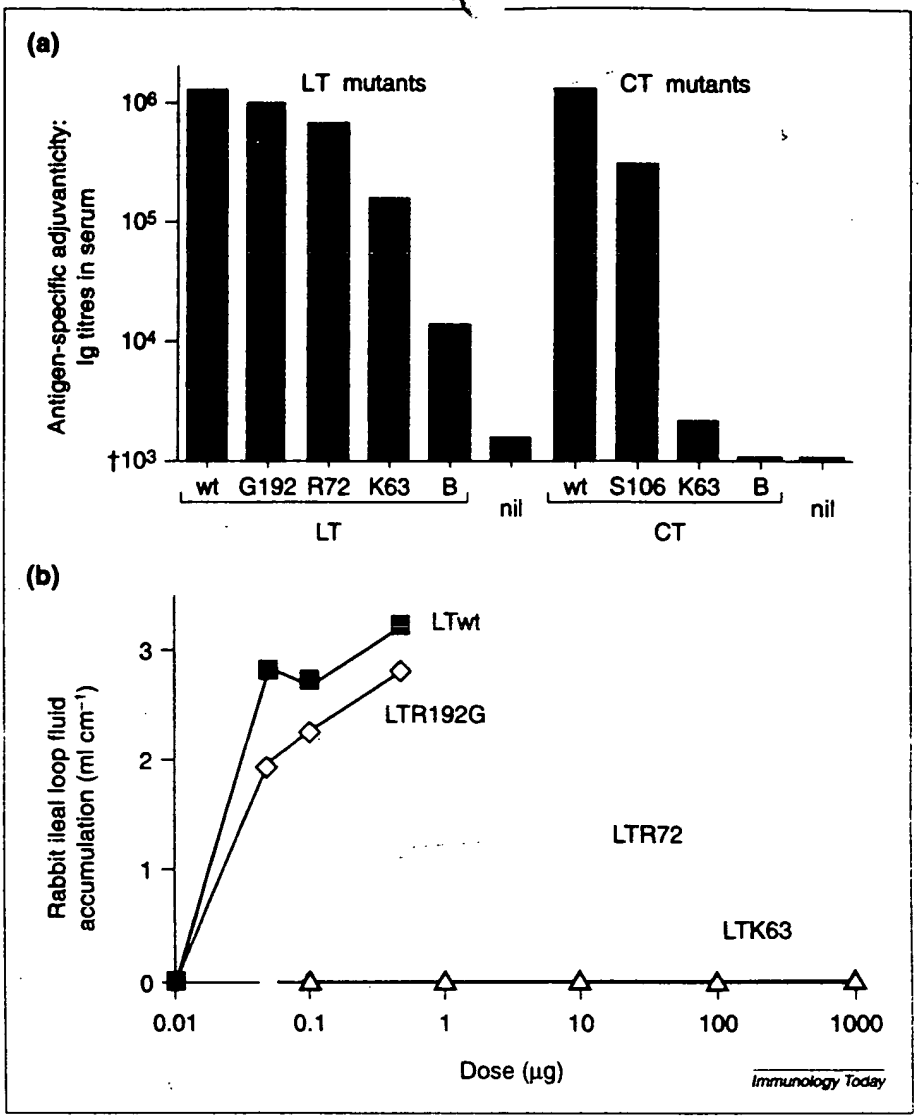


Fig. 3. *(a) Immunoglobulin (Ig) immune response to bystander antigens in sera of mice immunized intranasally with wild-type (wt) cholera toxin (CT) and heat-labile enterotoxin (LT) and their genetically detoxified derivatives as adjuvants. Results are shown as mean titres of antigen-specific antibodies. (b)* In vivo *toxicity in the rabbit ileal loop assay. Toxicity is expressed as the fluid accumulation (ratio of the amount of fluid collected in each loop to the length of the loop) induced by different amounts of LT and its genetically detoxified derivatives.*

Recombinant A subunit

An alternative approach to separate the adjuvant activity of LT and CT from toxicity has been the use of the A subunit alone. The A subunit of CT has been expressed as a fusion protein with a B-cell targeting moiety and the two Ig-binding domains (DD) of staphylococcal protein A, and hence called CTA1–DD fusion protein. This molecule retains the adjuvant activity of CT by directing the enzymatic activity of the A subunit to B cells and possibly other antigen-presenting cells[57]. Enzymatically inactive CTA1–DD derivatives fail to induce an adjuvant response following systemic immunization showing that the adjuvant effect of CTA1–DD depends on the enzymatic activity[58]. However, whether the enzymatic activity of these fusions is also essential for mucosal adjuvanticity has not yet been investigated.

In other experiments, both the His-tagged form of LTA, and the His-tagged form of the enzymatically inactive derivative LTA-K112 [LTA$(His)_{10}$ and LTA-K112 $(His)_{10}$, respectively] have been reported to retain the mucosal adjuvant properties of the wild-type toxin, suggesting that in this case the adjuvant effect is independent from ADP-ribosylation[37]. The mechanism by which a His-tagged A subunit can be internalized in the absence of a receptor binding domain is unclear. It is possible that the polycationic histidine peptide tail could provide a non-specific cell binding activity[59].

Non-toxic mutants act also as oral adjuvants

Most of the results so far described were obtained in mice by using the intranasal route for immunization. This route is very convenient because immunogenicity and adjuvanticity can be induced by using as little as a fraction of a microgram of antigen and adjuvant. Whether the conclusions so far reached are valid also for the oral route remains unresolved in the literature. Oral immunization usually requires large amounts of antigen (100–5000 μg) and adjuvant (at least 50 μg), and therefore most experiments are technically compromised by the potential involuntary intranasal contamination with small fractions of the vaccine during oral immunization. A second factor especially critical for oral adjuvanticity is the structural

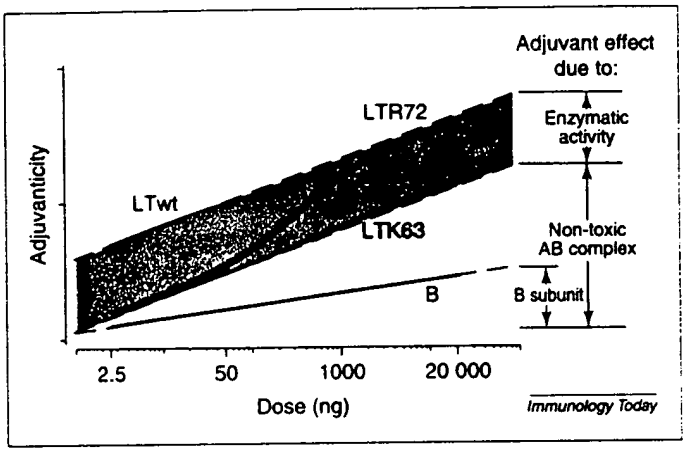


Fig. 4. *The results of a dose-response experiment that show the relative contribution of the B subunit, the non-toxic AB complex and the enzymatic activity to adjuvanticity.*

stability and protease sensitivity of both the mutants and the bystander antigen. This can influence the *in vivo* persistence of the holotoxin structure and therefore its activity. Recent studies taking into account the above technical problems, have come to the conclusion that LT, LTG192 and the non-toxic LT derivative LTK63, act as oral adjuvants to varying degrees. The B subunit alone is less successful as an adjuvant via this route[60].

Effects on the immune system

The literature describing the behaviour of LT and CT on the mucosal immune system is extensive and in many places contradictory. CT-mediated adjuvanticity appears to be accompanied by a preferential activation of T helper 2 (Th2)-type $CD4^+$ cell populations. This comes from the observation that mucosal immunization with antigens plus CT induces increased production of interleukins (IL) 4, 5 and 10, the predominant production of immunoglobulin G1 (IgG1) isotype and induction of antigen-specific IgE (Ref. 6). More recently, this polarization of the immune response towards a Th2 functional phenotype has been shown to be caused by the ability of CT to inhibit both the production of IL-12 p70 and the expression of the β1 and β2 chains of the IL-12 receptor. This might lead to the functional suppression of Th1 cell differentiation and to polarization towards a Th2-type response[61]. The ability of CT to polarize the immune response towards Th2 and to induce a selective upregulation of the B7-2 expression[62] is also maintained by enzymatically inactive mutants such as CTK112 (Ref. 63).

This polarization in the T-cell response is much less pronounced when LT is used as a mucosal adjuvant, with both Th1 and Th2 cells being activated[5]. In addition, recent studies suggest that LT mutants with one single amino acid substitution in the A subunit have different behaviours in the activation of the $CD4^+$ cell subpopulation. In these studies, the fully non-toxic LTK63 mutant promoted T-cell responses with a mixed Th1–Th2 profile, whereas the LTR72 mutant, which retained residual enzymatic activity, induced a more polar-

The polarization of the immune response seems to be affected not only by the different adjuvant molecules used, but also by the route of immunization[64].

Conclusions

Binding to mucosal receptors is a danger signal

Receptor binding is necessary for LT and CT to induce a mucosal immune response. This suggests that receptor binding is sufficient to differentiate LT and CT from the thousands of other molecules that associate with the mucosal surfaces without inducing a similar response. This observation is likely to be generally applicable to every molecule.

Mucosal surfaces provide a physical barrier between the external environment and the body, and the substances that come into contact with the mucosae usually do not closely interact with them. Exceptions are the small molecules that interact with the receptors for odours and taste and the small-molecular-weight peptides that are taken up in the gut following digestion of the proteins present in the food. Therefore, it makes sense for the body to mount a vigorous immune response against every molecule that actively binds to mucosal surfaces. In fact, this might be a signal that a molecule is trying to behave abnormally and therefore is potentially dangerous. Several reports that describe the mucosal immunogenicity of proteins that bind to mucosal receptors support the above conclusion. However, binding to the mucin layer of the gut rather than receptors on cells might not be sufficient to induce an immune response. Hence, some molecules with binding activity might not actually reach immune inductive sites or might be presented inappropriately, resulting in them being treated as environmental.

The surprising finding that some enzymatically inactive mutants are good mucosal adjuvants, whereas other mutants are not, can be explained by several factors: (1) their stability *in vivo*; (2) the efficiency of the ER targeting retention sequence (KDEL for CT and RDEL for LT)[65,66]; and (3) the different specificity of the receptor-binding site. The differential binding of LT and CT to different receptors might target the two molecules to different cell populations, thus changing their adjuvant effect. Because the receptors might be present in different tissues and in different animal species, we should consider the data reported here as relating only to intranasal delivery in the mouse. Therefore, it should not be too surprising if future studies will show CT mutants to be better adjuvants than LT mutants in different animal species or if delivered by different routes.

The n n-toxic AB complex and enzymatic activity in adjuvanticity

The availability of molecularly defined mutants has enabled the relative contributions of the B subunit, the non-toxic AB complex and the enzymatic activity to adjuvanticity to be studied. A dose response curve comparing LTK63, LTR72, wild-type LT and LTB as adjuvants showed that the B subunit is a poor adjuvant at all doses (although some adjuvanticity is present at very high doses), whereas

that is dose-dependent (Fig. 4). By contrast, the enzymatic activity provides a dose-independent adjuvant effect above a certain threshold of activity. This threshold is reached at 2.5 ng with LT and at approximately 1 μg with LTR72.

The adjuvant activity of the non-toxic AB complex might derive from properties of the A subunit, such as the binding to ARF factors or the ability to interact with the vesicular transport system. This could allow antigens present in LT-containing endosomes to reach the Golgi and the ER, thus facilitating their interaction with antigen-presenting systems. The marked dose dependence of the adjuvant activity of enzymatically inactive derivatives of LT and CT is enough to explain most of the controversial results present in the literature. Adjuvanticity can be demonstrated for virtually every molecule by increasing the dose used. To make sure that the best mutants are finally selected for human use and that scientifically sound conclusions are reached, similar doses should be compared in future studies.

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Eotaxin: from an eosinophilic chemokine to a major regulator of allergic reactions

Jose Carlos Gutierrez-Ramos, Clare Lloyd and Jose Angel Gonzalo

Eotaxin has a variety of effects on several cell types that are involved in the allergic inflammatory response. Here, Jose Carlos Gutierrez-Ramos and colleagues review the chemotactic effects of eotaxin on eosinophils and T helper type 2 cells, its differentiation and migration effects on mast cells and its actions on progenitors and mature cells in the bone marrow.

The elegant identification of eotaxin, a peptide with eosinophil chemotactic activities[1,2] opened the door to our understanding of the molecular basis for the highly selective eosinophil accumulation seen during allergic reactions. The molecular cloning of the gene encoding eotaxin[3–5], and the subsequent study of its sequence revealed that it belonged to a family of peptide chemoattractants termed chemokines. The chemokines are peptide ligands for the seven-transmembrane G-protein-coupled receptors expressed on leukocytes, which elicit changes in adhesiveness, cell motility and chemotaxis[6]. Based on function and sequence homologies, it was evident that eotaxin belonged to the CC subfamily of chemokines[3–5]. The CC subfamily of chemokines, which includes monocyte chemoattractant proteins (MCPs), RANTES and macrophage inflammatory proteins (MIPs) among others, acts predominantly on monocytes, lymphocytes and non-neutrophil granulocytes (reviewed in Ref. 6).

The protein product of the eotaxin gene was shown to be a very potent and efficacious chemoattractant for eosinophils *in vivo* and *in vitro*[3–5]. It is present and its synthesis regulated during allergic reactions and other pathological processes in which eosinophils are thought to play a role[7–11]. These clues were expeditiously followed by several groups, whose efforts resulted in the cloning of the gene encoding the eotaxin receptor, CCR3 (Refs 12, 13).

The identification of a chemotactic factor for eosinophils and its receptor was only the beginning of a series of findings that raised eotaxin to a unique position among the players in allergic inflammation

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Rapid note

Immunostimulatory activity of LT-IIa, a type II heat-labile enterotoxin of *Escherichia coli*

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Abstract

Certain bacterial molecules potentiate immune responses to parenterally administered antigens. One such molecule that has been intensely investigated is cholera toxin, a type I heat-labile enterotoxin produced by the Gram-negative bacterium *Vibrio cholerae*. Immunization with a mixture of a foreign antigen and cholera toxin enhances the immune response to the antigen. Similar adjuvant activity is associated with LT-I, a closely related type I heat-labile enterotoxin produced by *Escherichia coli*. The adjuvant activities of LT-IIa, a member of the type II heat-labile enterotoxins produced by *E. coli*, have not been described. LT-IIa and CT differ significantly in amino acid sequence of the B polypeptides and in receptor binding affinity. In this study, rats were subcutaneously immunized with fimbrillin, a protein isolated from the bacterium *Porphyromonas gingivalis*, and with fimbrillin in combination with LT-IIa, the prototypical type II enterotoxin. Previous studies documented that fimbrillin administered alone is a poor immunogen. Animals immunized with the mixture of fimbrillin and LT-IIa produced high titers of specific IgG antibody directed against fimbrillin. Anti-fimbrillin antibody titers in sera from animals receiving the combination of LT-IIa + fimbrillin were comparable to those obtained from sera of animals immunized with cholera toxin + fimbrillin. The results of these experiments demonstrate that LT-IIa exhibits an adjuvant activity that is equal to that of cholera toxin. Recombinant methods have been established for producing large amounts of LT-IIa, an advantage that will likely provide an economic impetus to consider incorporating the enterotoxin as an immunostimulatory agent in future vaccines.

Keywords: Adjuvant; Enterotoxin; Fimbrillin; LT-IIa

The heat-labile enterotoxins produced by *Escherichia coli* and *Vibrio cholerae* belong to a family of proteins that are related in structure and function [1]. Members of this family include cholera toxin (CT) expressed by *V. cholerae* and the enterotoxins LT-I and LT-IIa, expressed by enterotoxigenic strains of *Escherichia coli* [2]. All members of the family are oligomeric proteins, composed of a single A subunit that is non-covalently bound to a pentameric array of B polypeptides [2]. Treatment of holotoxin with trypsin cleaves the A polypeptide into two fragments joined by a disulfide bond [3]. The A1 fragment is a potent ADP-ribosyl transferase that is responsible for the enterotoxigenic effects, while the A2 fragment derived from the C-terminal end of the A polypeptide non-covalently binds the A subunit to the B pentamer. The B pentamer mediates binding of the enterotoxin to gangliosides [4], a heterogeneous family of sugar-containing lipids on the surface of mammalian cells [5]. Each enterotoxin

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has a unique ganglioside-binding specificity [4]. CT binds to ganglioside GM1 (GM1), whereas LT-I binds to GM1 and several glycoproteins [6]. LT-IIa binds with strong affinity to ganglioside GD1b (GD1b), and with less affinity to gangliosides GD1a, GM1, GT1b, GQ1b, and GD2 [4]. The property to recognize different receptors likely enables the enterotoxins to bind to the surface of different cells or tissues [7].

CT, LT-I and LT-IIa have been purified and the operons encoding the A and B subunit genes have been cloned and sequenced (reviewed in [2]). Comparisons of the predicted amino acid sequences show that the A polypeptides of CT, LT-I, and LT-IIa are similar (at least 50% identity) [2]. In contrast, the B polypeptides of CT and LT-I, while highly homologous to each other (at least 80% identity), have little or no homology to the B polypeptides of LT-IIa (less than 14% identity). The differences in amino acid sequences are sufficient to produce antigenic heterogeneity between the members of the family. Antisera against CT and LT-I will not react with LT-IIa, and vice versa [2]. Based on differences in immunoreactivity, the family of heat-labile enterotoxins was divided into two classes, the type I and type II enterotoxins. Type I enterotoxins include CT and LT-I, while LT-IIa, and a related enterotoxin, LT-IIb, comprise the type II class of heat-labile enterotoxins.

CT is a highly immunogenic protein that stimulates potent secretory and systemic immune responses [8,9] and is known to have adjuvant activity. Both secretory and humoral immune responses to foreign antigena are enhanced when an animal is immunized with a mixture of antigen and CT [10]. Whereas adjuvant activity is most pronounced when CT holotoxin is employed, it has been demonstrated that immunization with the non-toxic B pentamer also elicits an enhanced immune response to foreign antigens [10,11]. These studies suggest that the immunomodulatory properties of CT are associated, in part, with GM1-binding activity.

While the adjuvant activity of CT has been established, the potential of the type II heat-labile enterotoxin LT-IIa for enhancing immune responses has not been investigated. We hypothesized that with its binding affinity for a variety of gangliosides, LT-IIa may bind to and stimulate cells of the immune system that are not bound or stimulated by CT or may bind to the same cells in larger quantities. In either case, we predicted that the adjuvant activity of LT-IIa, if present, would be equal or greater than that of CT.

To determine if LT-IIa has adjuvant activity and to compare that potential adjuvant activity with that of CT, conventional Sprague–Dawley rats were immunized with fimbrillin in the presence or absence of purified CT or LT-IIa (a gift from Dr R.K. Holmes). Fimbrillin is the major polypeptide comprising the fimbrial structure of the Gram-negative oral pathogen *Porphyromonas gingivalis* [12]. Parenteral immunization with fimbriae elicits only a weak anti-fimbrillin antibody response in the rat model [13]. For these experiments, five groups of rats ($n = 4–8$ rats per group) were immunized with purified fimbrillin [13], CT, LT-IIa, CT + fimbrillin, or LT-IIa + fimbrillin. Rats ($n = 6$) that received sham immunizations of buffer without enterotoxin or fimbrillin served as a control group. Solutions used for immunizations contained enterotoxin and fimbrillin at concentrations of 10 μg/ml and 100 μg/ml, respectively. One hundred microlitres of the immunizing solutions were administered subcutaneously at each of two sites. The amounts of enterotoxin (2 μg) and antigen (20 μg) used were comparable to amounts of antigen used in similar immunization studies [7,13]. Phosphate-buffered saline (PBS) was used as the diluent in all cases. Rats were immunized at day 0 and at day 28 with enterotoxin, fimbrillin, or enterotoxin + fimbrillin, as appropriate. Forty-two days after the primary immunization, animals that were immunized with fimbrillin, CT + fimbrillin, and LT-IIa + fimbrillin received a second booster immunization of fimbrillin. The remaining groups (CT, LT-IIa, and the control group) received sham immunizations of PBS at day 42. A blood sample was collected from the lateral tail vein of each rat prior to the initial immunization (day 0) and at days 14, 28, 42 and 49. Serum was separated from the clotted blood by centrifugation.

Particle concentration fluorescence immunoassay (PCFIA) was used to measure the amount of anti-fimbrillin antibodies in serum samples [14]. In brief, purified fimbrillin was covalently bound to carboxylated polystyrene beads (IDEXX, Westbrook, ME) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St. Louis, MO). Titration studies were used to determine the amount of fimbrillin that was required in the binding reaction to saturate the surface of the beads with the antigen (data not shown). Fimbrillin-coated beads were incubated with a 1:100 dilution of serum, washed with PBS to remove unbound antibody, and incubated with a 1:35 dilution of fluorescein isothiocynate (FITC)-labeled affinity purified goat anti-rat IgG immunoglobulin (Kirkegaard and Perry, Gaithersburg, MD). After extensive washing to remove unbound FITC-labeled antibodies, fluorescence of the treated beads was measured at 485/535 nm in an automated fluorimeter (IDEXX, Westbrook, ME). Anti-fimbrillin antibody titers in the sera were expressed as relative units of fluorescence (RFU). Previous studies demonstrated that the level of fluorescence detected by PCFIA is directly correlated with the amount of antibody bound to the antigen-sensitized beads [13]. The technique is highly quantitative [13]. The amount of antibodies in the serum samples directed against LT-IIa and CT was measured by an enzyme-linked immunosorbent assay (GD1b-ELISA) [15]. Rat hyperimmune

antiserum raised against purified fimbrillin was used as a positive control in all experiments. Data from different groups were compared using an analysis of variance test to determine significance.

As expected, rats immunized with CT + fimbrillin showed a much stronger response to the fimbrial antigen than did rats immunized with fimbrillin alone ($P < 0.001$) (Fig. 1). By day 42, the anti-fimbrillin response elicited by immunization with fimbrillin was meager (4072 ± 559 RFU) (data are reported as the mean RFU $\pm$ one standard error of the mean). Rats immunized with CT + fimbrillin responded strongly on day 42 and produced high titers of anti-fimbrillin antibody (13372 ± 1539 RFU). Seven days after a booster immunization with fimbrillin (day 49), the immune response to the antigen was enhanced further in the CT + fimbrillin group (35698 ± 2757 RFU). Animals boosted with fimbrillin alone showed a small enhancement in specific antibody responses (13782 ± 2743 RFU), but the enhancement was not as dramatic as with the animals that had been previously immunized with fimbrillin + CT or fimbrillin + LT-II.

Analysis of the sera from rats immunized with fimbrillin and LT-IIa + fimbrillin demonstrated that LT-IIa has potent adjuvant activity (Fig. 1). By day 42, rats immunized with LT-IIa + fimbrillin developed a significantly higher titer of anti-fimbrillin antibody than

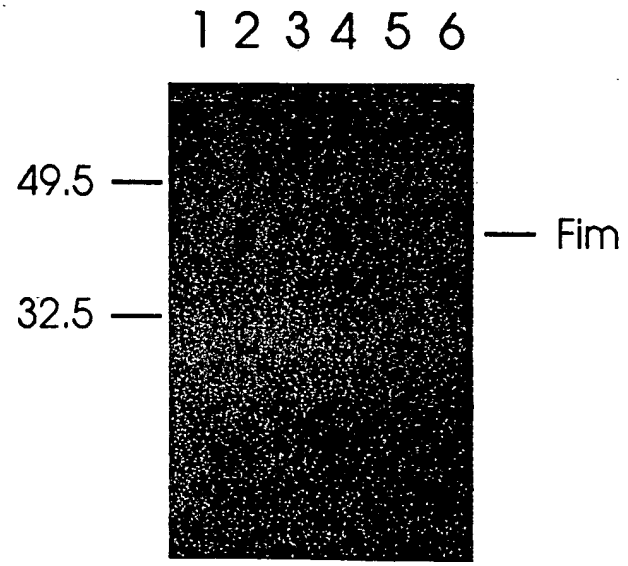

Fig. 1. Immunoblot analysis of the enterotoxin-enhanced immune response to fimbrillin. Purified fimbrillin was immunoblotted with a 1:50 dilution of antisera collected on day 49 from rats immunized with: 1, CT; 2, CT + fimbrillin; 3, LT-IIa; 4, LT-IIa + fimbrillin; 5, fimbrillin; and 6, PBS (sham immunization). In each case, the antiserum for the immunoblot was obtained from a rat in the relevant group that had a specific antibody titer that most closely approximated the mean antibody titer of the group, as determined by PCFIA. Immunoblots were developed using goat anti-rat IgG (BioDesign, Kennebunk, MA) and 4-chloro-1-napthol (Biorad, Richmond, CA).

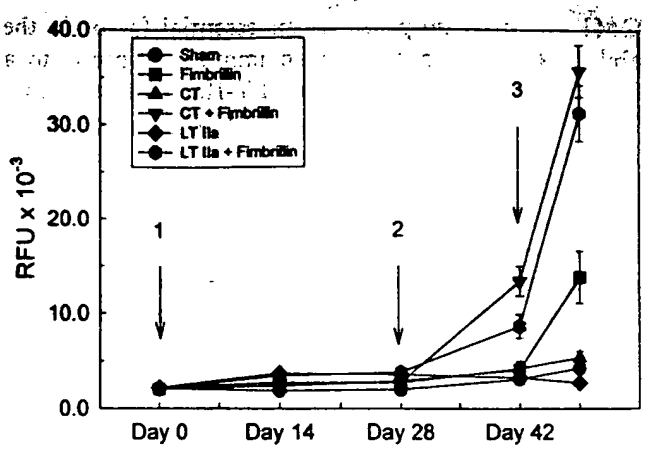

Fig. 2. Enhanced IgG-specific immune response to fimbrillin after co-immunization with CT or LT-IIa. Rats were immunized with fimbrillin, LT-IIa, CT, fimbrillin + CT, and fimbrillin + LT-IIa. Symbols for each group are noted in the box. IgG antibody titers in the sera collected on days 0, 14, 28, 42 and 49 were measured by PCFIA. Time points at which immunizations were given are denoted by arrows: 1, initial immunization; 2, second immunization; 3, booster immunization using purified fimbrillin (given only to groups previously immunized with fimbrillin, fimbrillin + CT, or fimbrillin + LT-IIa). RFU—relative fluorescence units. Bars indicate one standard error of the mean.

did rats immunized with fimbrillin without the enterotoxin (8644 ± 1242 RFU vs 4072 ± 559 RFU, respectively; $P < 0.001$) (Fig. 1). By day 49 after booster immunizations, the differences between the two groups increased (31226 ± 2969 vs 13782 ± 2743 RFU, respectively; $P < 0.001$). Comparisons of the levels of antibodies produced by rats immunized with CT + fimbrillin to the levels of antibodies produced by rats immunized with LT-IIa + fimbrillin showed that the two groups of rats had equal anti-fimbrillin antibody titers ($P = 0.31$).

To confirm that the immunoreactivity in the serum samples detected by PCFIA was directed against fimbrillin, pooled sera from each group were diluted 1:50 and analyzed by Western blotting (Fig. 1). Sera collected on day 49 from rats immunized with fimbrillin + CT and with fimbrillin + LT-IIa showed a strong response to purified fimbrillin. Although sera from rats immunized with fimbrillin without enterotoxin had detectible immunoreactivity by PCFIA, the amount of anti-fimbrillin antibody in a 1:50 dilution was below the level of detection of the immunoblot (Fig. 2). The rats in all groups immunized with enterotoxins produced high levels of anti-enterotoxin antibodies. Sera collected from animals in the CT and the CT + fimbrillin group had high titers of CT-specific antibodies and sera from animals in the LT-IIa and LT-IIa + fimbrillin groups had high titers of anti-LT-IIa antibodies (data not shown).

The data demonstrate that LT-IIa and CT are equally potent adjuvants in this rat model. Affinity for

GM1 has been proposed as an essential factor in the ability of CT to potentiate an immune response to a foreign antigen [11]. Although LT-IIa binds to GM1, GM1 is not the preferred receptor [4]. It is not clear whether the adjuvant activity of LT-IIa is a result of low affinity binding of the enterotoxin to GM1 or to high affinity binding of the enterotoxin to alternative gangliosides on host cells or tissues involved in immune responsiveness. Mutant LT-IIa enterotoxins with altered ganglioside-binding specificities and mutant LT-IIa enterotoxins with no detectible ganglioside-binding activities have been reported [15]. It will be interesting to employ these mutant toxins in experiments similar to those reported here to determine in a direct manner the importance of ganglioside-binding in induction of adjuvant activity.

This report establishes that LT-IIa is a potent immunostimulatory molecule. Further studies of LT-IIa must be done to define the factors that are most important in potentiating immune responses. The ability to produce very large amounts of LT-IIa using established recombinant methods [15] should provide an economic impetus for the use of the enterotoxin in the development and production of future vaccines.

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Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of *Escherichia coli* heat labile enterotoxin in attenuated salmonellae

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Two overlapping T-cell sites of the nucleocapsid antigen (HBc) of Hepatitis B Virus (HBV) (amino acids (aa) 120–140) and a B-cell epitope of the pre-S(2) region of the HBV surface antigen (aa 133–140) were expressed as a fusion protein with the subunit B of* Escherichia coli *heat labile enterotoxin (LT-B) in attenuated salmonellae (*aroA Salmonella dublin *SL1438). When Balb/c (haplotype H-2^d) mice were fed salmonellae expressing LT-B or the LT-B/HBV fusion protein they developed serum IgG anti-LT-B antibodies and splenic cells reactive to LT-B. C57BL/10 (H-2^b), in contrast, showed anti-LT-B antibody titres, but no splenic cell priming to LT-B. Neither in Balb/c nor in C57BL/10 mice could an antibody response to the fused HBV antibody binding site be demonstrated. In C57BL/10, however, an HBc T-cell epitope fused to LT-B primed a splenic cell response to an analogous synthetic peptide (HBc aa 121–145) in four out of five mice after three oral immunizations. This is the first description of the priming of a cellular immune response to a defined heterologous epitope expressed in attenuated salmonellae and delivered by the oral route.

Keywords: Hepatitis B; T-cell epitope; fusion protein; *E. coli* enterotoxin

Introduction

Infection with hepatitis B virus (HBV), a small DNA virus (for reviews see refs 1, 2, 3) is the cause of acute and chronic liver disease in man and HBV continues to be one of the most important pathogens on a world-wide scale with over 200 million chronic carriers[4]. Despite the existence of safe and efficient plasma-derived and recombinant vaccines[5], availability and cost still hamper vaccination programmes similar to the successful immunization against smallpox virus. Existing vaccines are based on HBV surface antigens. Immunization with recombinant HBV core antigen (HBc) has also been shown to provide some degree of protection against HBV infection[6,7], probably mediated by cellular immune response mechanisms, since HBc is an internal viral antigen. HBc particles elicit both T-cell dependent and T-cell independent antibody responses and a strong cellular immune response[8]. T-cell epitopes of HBc have been mapped in the murine system[9], but it is not known which epitopes mediate protective immunity. It has recently been demonstrated that immunization with recombinant (human) HBc induces a partial protection in woodchucks against infection with a closely related hepadnavirus, woodchuck hepatitis virus (WHV) (F. Schödel, K. Manneck, K. Fuchs, H. Will and M. Roggendorf, unpublished). Therefore HBc amino acid sequences mediating protective immune responses are probably in the regions conserved among WHV and HBV (for a comparison of amino acid sequences see Ref. 1). An ideal vaccine would comprise protective T-cell epitopes for a longlasting memory and elicit virus neutralizing antibodies against defined epitopes. It would be an additional advantage if this vaccine could be given orally and would combine stability and ease of production. Invasive but non-pathogenic *aroA* salmonella strains have been developed[10,11] which confer immunity to salmonella infections and are suitable as carriers for heterologous antigens (Refs 10, 12; for a review see Ref. 13). It has been shown that oral immunization with recombinant salmonellae expressing a circumsporozoite antigen can induce protective, possibly cell-mediated, immunity against *Plasmodium berghei* infection in mice[14]. Antibodies against HBV surface antigen epitopes could be elicited by the same route when the epitopes were expressed in salmonellae as flagellin inserts[15]. The development of an expression system which allows the stable expression of foreign epitopes as fusion proteins with the subunit B of *Escherichia coli* heat labile enterotoxin (LT-B) was described previously[16]. These fusion proteins expressed in SL1438, an *aroA Salmonella dublin* auxotroph, induce high level serum IgG antibodies to LT-B when fed orally to Balb/c or C57BL/10 mice and splenic T-cells reactive with LT-B when fed to Balb/c[17]. Serum antibodies to the fused HBV antibody binding sites could not be detected. It was described

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recently that two HBc T-cell epitopes (HBc amino acids (aa) 120–140) fused to an antibody binding site of the middle HBV surface protein in a synthetic peptide elicited both a strong H-2 restricted T-cell response after immunization in mice and provided T-cell help for the attached B-cell epitope (HBV pre-S(2) amino acids 133–140)[18]. It has been possible to stably express the same HBc T-cell epitopes and the B-cell epitope as a fusion protein with the carboxy terminus of LT-B (expression plasmid pFS20) in the vaccine strain SL1438[17]. Here the cellular immune response in mice fed with SL1438(pFS20) against one of the two HBc T-cell epitopes and against LT-B, is described.

Materials and methods

Bacteria and plasmids

The *aroA S. dublin* vaccine strain SL1438[10] was kindly provided by Bruce Stocker (Stanford). The construction of expression plasmids pFS2.2 (expressing LT-B + eight carboxyterminal amino acids from a polylinker) and pFS20 (HBc aa 120–145 and HBV pre-S(2) aa 133–140, fused to the carboxyterminus of LT-B) has been described previously[17]. Maintenance and growth conditions of bacteria were as described before[16,17].

Immunization

As described previously[16,17] 10–12 female Balb/c or C57BL/10 mice were fed with $\approx 10^{10}$ colony forming units (c.f.u.) of SL1438(pFS20) [group M, C57BL/10 and group N, Balb/c] or SL1438(pFS2.2) [group O] by orogastric tube. Blood was drawn 10 days after the first immunization and the animals were immunized a second time. Again blood was drawn after 10 days a set of animals was killed and the spleens were removed for establishing T-cell cultures. A second set of animals was immunized a third time and blood and spleen cells removed 14 days after the third immunization.

Cell culture and antibody determination

Spleens were aseptically removed and carefully dissected. Splenic cells were separated over a Ficoll/Isopaque gradient and cultured in RPMI-Click (Gibco) supplemented with 0.5% syngeneic serum in microtitre plates. The cell density was adjusted to 5×10^5 cells/well and affinity purifed LT-B (kindly provided by John Clements) to a concentration of 0.1 μg, 1 μg or 10 μg ml^{-1} or a synthetic peptide spanning HBc amino acids 121–145 (synthesized by automated synthesis, >90% pure by HPLC analysis) to a concentration of 1 μg, 10 μg or 100 μg ml^{-1} was added. The HBc synthetic peptide was weighed, solubilized by ultrasound, sterile filtrated and diluted before use. The indicated concentrations reflect the amount of peptide weighted in, actual concentrations in the cell culture wells may be lower due to low solubility of the peptide and loss during sterile filtration. The splenic cells were cultivated in a humid atmosphere at 37°C and 5% CO_2 for 5 days. In the last 18 h of culture, the cells were labelled with 0.5 μCi ^{3}H-thymidine (Amersham), harvested and counted in a liquid scintillation cocktail (Rackbeta). Background values of cells cultivated in the absence of antigen were determined and automatically subtracted. Anti-LT-B and anti-HBV pre-S(2) antibodies in the serum were determined at a 1/100 serum dilution by GM1-ELISA and anti-peptide (HBV pre-S(2) aa 133–150) ELISA, respectively, as described[17]; the second antibody was goat anti-mouse IgG (H + L), peroxidase coupled (Medac, Hamburg), substrate orthophenyldiamine.

Results

The cellular and humoral immune responses of mice fed with salmonellae expressing LT-B or a LT-B/HBV fusion protein were analysed. As previously demonstrated for LT-B alone and other LT-B fusion proteins[17], mice fed with salmonellae expressing either LT-B or the LT-B/HBc/HBV-pre-S(2) fusion protein developed serum IgG antibodies against LT-B (*Figure 1, a–c*). In Balb/c

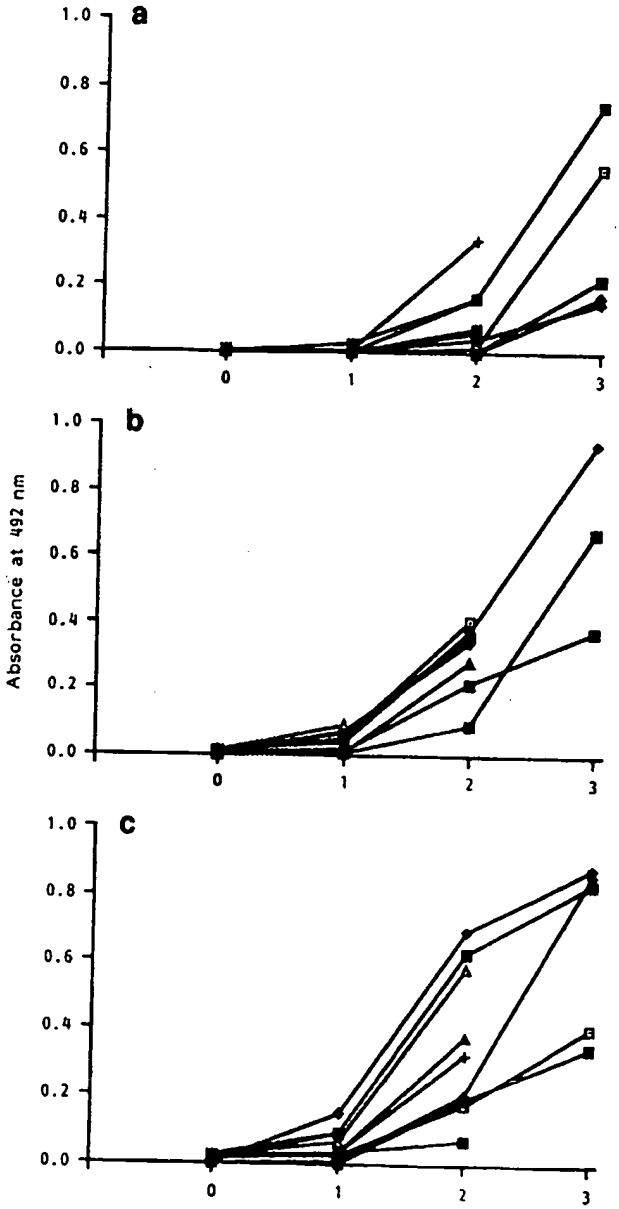
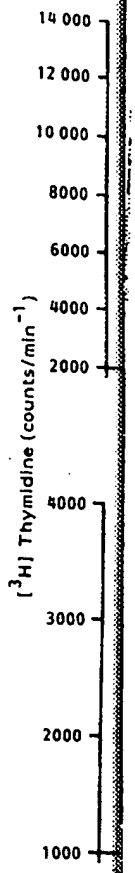


Figure 1 Serum IgG anti-LT-B antibodies after oral immunization of C57BL/10 mice with SL1438 (pFS20) (a), or Balb/c mice with SL1438(pFS20) (b) or SL1438(pFS2.2) (c). The means of duplicate absorbance measurements at 492 nm in a GM1 ELISA are indicated: 0 = pre-immune, 1 = 10 days after the first immunization, 2 = 10 days after the second immunization, 3 = 14 days after the third immunization. Lines connect values of individual sera



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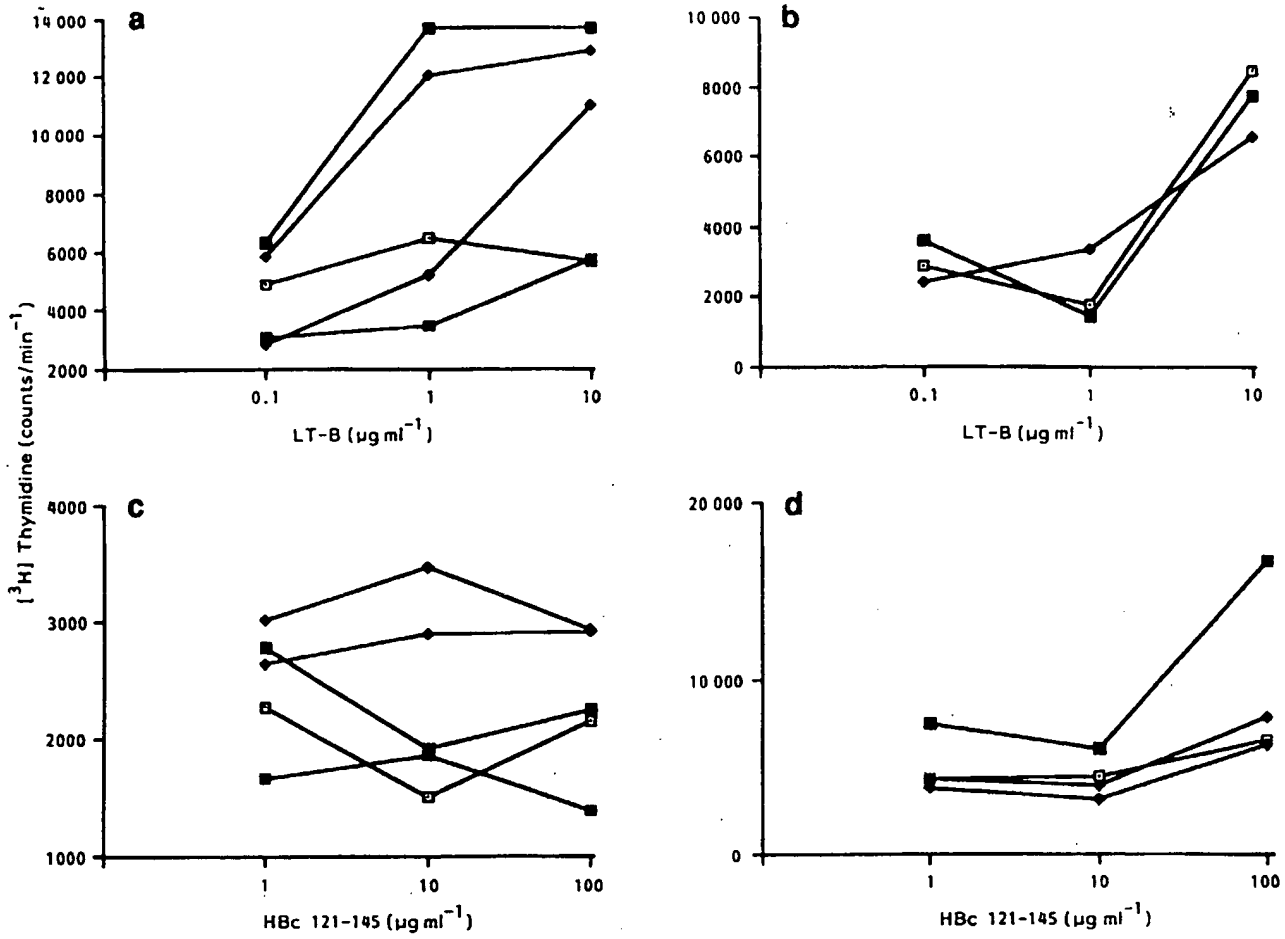

Figure 2 Stimulation of splenic cells in the presence of LT-B (a,b) or a synthetic peptide from the HBc region aa 121–145 (c,d). Mean counts min^{-1} of triplicate cultures labelled with ^{3}H-thymidine are indicated. Values of individual animals are connected. (a), group N (Balb/c, SL1438(pSF20)), (b) and (c), group O (Balb/c, SL1438(pFS2.2)), (d), group M (C57BL/10, SL1438(pFS20), here only the values of four out of five animals showing thymidine incorporation are plotted

mice, a higher rate of seroconversion was observed after the second oral immunization (*Figure 1b,c*) than in C57BL/10 mice (*Figure 1a*). After the third immunization all animal sera analysed showed detectable levels of serum anti-LT-B IgG. Antibodies against the carboxyterminally fused HBV-pre-S(2) B-cell epitope could not be detected in either Balb/c or C57BL/10 mice by anti-peptide ELISA (data not shown). After the third oral immunization, splenic cells reactive to LT-B could be observed in Balb/c mice fed with LT-B or LT-B fusion protein expressing salmonellae (*Figure 2a,b*). In contrast, in C57BL/10 mice no splenic cells primed against LT-B were detected (data not shown). Apart from a possible major histocompatibility complex restriction for LT-B this may be a hint that LT-B is capable of inducing antibodies by a T-cell independent mechanism, as antibodies against LT-B were readily detected in C57BL/10. In Balb/c an LT-B dose dependent increase in ^{3}H-thymidine incorporation was observed whether the animals were immunized with salmonellae expressing LT-B alone (group O, *Figure 2b*) or the fusion protein (group N, *Figure 2a*). The stimulation seems, however, higher in the group that received salmonellae expressing only LT-B (*Figure 2b*). Only in mice with an H-2^b genetic background (C57BL/10) was a stimulation of splenic cells in the presence of the HBc analogous synthetic peptide observed (*Figure 2d*); in Balb/c mice (H-2^d) SL1438(pFS20) did not prime a response (not shown). The HBc 121–145 synthetic peptide induced no proliferative response in splenic cells of Balb/c fed with SL1438(pFS2.2) as a negative control (*Figure 2c*). Similarly, no *in vitro* proliferative response to the synthetic peptide was observed in splenic cells of unprimed C57BL/10 or of C57BL/10 fed with salmonellae expressing LT-B alone (not shown). This observation is consistent with the previously described H-2 restriction of the HBc T-cell epitope 129–140[9,18]. Primed T cells of the HBc synthetic peptide could be demonstrated in four out of five mice analysed after the third immunization with SL1438(pFS20).

Discussion

The data presented indicate that a heterologous T-cell epitope fused to the carboxyterminus of LT-B can be presented to the mouse immune system by orally given attenuated salmonella. To our knowledge this is the first time that it has been demonstrated that attenuated salmonella can induce an immune response to a defined heterologous T-cell epitope by the oral route. Heterologous B-cell epitopes fused to the carboxyterminus of LT-B, although they are accessible on the non-denatured protein, as tested by ELISA type assays with the respective poly- or monoclonal antibodies (data not

shown), do not induce a detectable B-cell response. The reason for the low B-cell immunogenicity of the fused sequences compared with LT-B alone is not known. It has recently been demonstrated that B-cell epitopes of the cholera toxin subunit B, highly homologous to LT-B, and of the HBV pre-(S) and S region inserted into a hypervariable sequence of H-1d phase-1 flagellin can be expressed in *aroA S. typhimurium* in an immunogenic form[15,19] and in the case of the HBV epitopes induce low serum antibodies by the oral route after four subsequent immunizations in a mouse model[15]. This approach could lead to the formation of neutralizing antibodies against HBV in man provided that the epitopes can be expressed in suitable *S. typhi* in an immunogenic form. Induction of T-cell responses to one or two T-cell sites may not suffice to induce protection in an outbred population. As the T-cell sites used here (HBc120–140) are particularly well conserved between HBV and WHV[1] they could play a role in inducing protective immunity. The finding reported here that a T-cell response can be induced by orally given *Salmonella* vaccine strains together with the successful induction of antibody responses to heterologous flagellin inserts expressed in salmonellae[15] paves the way for construction of recombinant bacteria that present a selection of artificially combined T-cell epitopes together with B-cell epitopes eliciting neutralizing antibodies, possibly on different structures within the same bacterium. Since the amount of heterologous antigen actually delivered to the host immune system may be critically affected by plasmid loss *in vivo* in the experiments described here and the presence of antibiotic resistance markers on plasmids used for *in vivo* vaccinations is not desirable, we have constructed *asd* plasmids expressing LT-B and LT-B/HBV fusion proteins and are currently testing their immunologic properties (F. Schödel, H. Will, T. Doggett and R. Curtiss, unpublished results).

These expression plasmids without antibiotic resistance markers are highly maintained in salmonella vaccine strains by complementation of a lethal mutation in the cell wall metabolism (Δ aspartate semialdehyde dehydrogenase [*asd*])[20]. Cells that lose the plasmids lyse and release their antigenic content. Also hybrid HBc/pre-S proteins which form particles[21] have now been successfully expressed in attenuated salmonellae (F. Schödel, unpublished results) and their immunogenicity is under investigation.

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Characterization of Hybrid Toxins Produced in *Escherichia coli* by Assembly of A and B Polypeptides from Type I and Type II Heat-Labile Enterotoxins

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The genes encoding the individual A and B polypeptides of the type I enterotoxin LTp-I and type II enterotoxins LT-IIa and LT-IIb were cloned and tested for complementation in *Escherichia coli*. Each gene encoding an A polypeptide was cloned into pACYC184, and each gene encoding a B polypeptide was cloned into the compatible plasmid Bluescript KS+. In addition, operon fusions representing all combinations of A and B genes were constructed in Bluescript KS+. Extracts from strains of *E. coli* expressing each combination of A and B genes, either from compatible plasmids or from operon fusions, were tested for immunoreactive holotoxin by radioimmunoassays and for toxicity by Y1 adrenal cell assays. Biologically active holotoxin was detected in each case, but the toxicity of extracts containing the hybrid toxins was usually less than that of extracts containing the wild-type holotoxins. The ganglioside-binding activity of each holotoxin was tested, and in each case, the B polypeptide determined the ganglioside-binding specificity. The A and B polypeptides of the type II heat-labile enterotoxins were also shown to form holotoxin in vitro without exposure to denaturing conditions, in contrast to the polypeptides of the type I enterotoxins that failed to form holotoxin in vitro under comparable conditions. These findings suggest that type I and type II enterotoxins have conserved structural features that permit their A and B polypeptides to form hybrid holotoxins, although the B polypeptides of the type I and type II enterotoxins have very little amino acid sequence homology.

The heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* constitute a family of proteins that are related in structure and function (22, 33). They are classified into two serogroups (9, 21, 37–39). Antisera against the enterotoxins in serogroup I do not neutralize the toxins in serogroup II, and vice versa. Serogroup I includes cholera toxin (CT) and the *E. coli* type I heat-labile enterotoxins (LT-I). LTh-I and LTp-I are antigenically cross-reacting variants of LT-I produced by *E. coli* strains from humans and pigs, respectively (6, 23). Serogroup II includes the *E. coli* type II heat-labile enterotoxins (LT-II), with antigenic variants designated LT-IIa and LT-IIb (14, 15, 19, 21, 37–39). Strains of *E. coli* that produce LT-II have been isolated from animals (water buffalo, cattle, pigs, etc.) and foods, particularly in Southeast Asia and South America, but they are rarely isolated from humans (14, 40). CT and LT-I function as virulence factors and cause diarrhea with cholera and enterotoxigenic *E. coli* infections, but the role of type II toxins in pathogenesis has not yet been demonstrated.

Toxins in the *V. cholerae* and *E. coli* heat-labile enterotoxin family have one A polypeptide and five B polypeptides, and quaternary structure of the holotoxins is maintained by noncovalent bonds between the polypeptides (12, 14, 16, 22, 43). Treatment of holotoxin with trypsin cleaves its A polypeptide into fragments A1 and A2, which remain joined by a disulfide bond, and fragment A2 mediates interaction of the A polypeptide with the B polypeptides. Holotoxin binds via its B polypeptides to surface-exposed sugars of gangliosides that function as specific receptors on eucaryotic cell membranes (11, 22). Both CT and LT-I bind preferentially to ganglioside GM1, but LT-I can also bind to glycoproteins (13). LT-IIa binds with greatest affinity to ganglioside GD1b, and LT-IIb binds preferentially to ganglioside GD1a (11). Binding of holotoxin triggers entry of fragment A1 into the target cells, in which it activates plasma membrane adenylate cyclase by catalyzing ADP ribosylation of the regulatory protein $G_{s\alpha}$. Biological effects of the heat-labile enterotoxins are believed to be mediated primarily by increased concentrations of cyclic 3′,5′-AMP (cAMP) in the target cells, but cAMP-independent effects of enterotoxins that may be mediated by prostaglandins or eicosanoids or by cross-linking of gangliosides in plasma membranes have also been described previously (10, 35).

The genes that encode the A and B polypeptides of CT, LTh-I, LTp-I, LT-IIa, and LT-IIb have all been cloned and sequenced (7, 27, 28, 32, 36, 37, 39, 45). Comparison of the predicted amino acid sequences for these representative type I and type II enterotoxins shows that their A1 fragments are most homologous (22, 36–39, 45); this homology presumably reflects the conserved ADP ribosyltransferase activities of the A1 fragments (26). The A2 fragments are much less homologous than the A1 fragments (36, 38). Within serogroup I or II, the B polypeptides are homologous, but B polypeptides of type I enterotoxins have little or no significant homology with those of type II (36, 38). The apparent lack of homology between B polypeptides of type I and type II enterotoxins is consistent with their different ganglioside-binding specificities, and the limited homology between A2 fragments of type I and type II enterotoxins may reflect coevolution of the A2 polypeptides with the specific B polypeptides to which they bind (22).

Assembly of type I holotoxins occurs after transport of their A and B precursor polypeptides into the periplasm and removal of their signal peptides (7, 16, 17, 32). The mature B polypeptides of type I enterotoxins can associate to form

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TABLE 1. Strains and plasmids used in this study

Plasmid or *E. coli* strain	Phenotype	Construction	Reference
Vectors and parental plasmids			
pACYC184	Nontoxinogenic	Clr^r Tet^r, P15A replicon	5
pBluescript	Nontoxinogenic	Amp^r, ColE1 replicon, P_{lac} (Stratagene)	41
pEWD299	LT-I A, LT-I B	LT-I operon from p307	7
pCP3727	LT-IIa A, LT-IIa B	LT-IIa operon from *E. coli* SA53	39
pCP4185	LT-IIb A, LT-IIb	LT-IIb operon from *E. coli* 41	31
pACYC184 constructions			
pTC900	LT-I A	1.3-kbp *Sma*I fragment from pEWD299	This study
pTC304	LT-IIa A	1-kbp *Pst*I fragment from pCP3727	This study
pTC601	LT-IIb A	1.58-kbp *Eco*RV-*Dra*I fragment from pCP4185	This study
pBluescript constructions			
pTC801	LT-I B	750-bp *Hin*dIII fragment from pEWD299	This study
pTC400	LT-IIa B	800-bp *Eco*RI-*Hpa*I fragment from pCP3727	This study
pTC700	LT-IIb B	1.0-kbp *Hin*dIII-*Pst*I fragment from pCP4185	This study
pTC5000	LT-I A, LT-I B	1-kbp *Bam*HI-*Eco*RI fragment from pTC900 and 3.6-kbp fragment from pTC801	This study
pTC200	LT-IIa A, LT-IIa B	2.2-kbp *Eco*RI-*Kpn*I fragment from pCP3727	This study
pTC101	LT-IIb A, LT-IIb B	1.68-kbp *Bgl*II-*Hpa*I fragment from pCP4185	This study
pTC1000	LT-I A, LT-IIa B	1.3-kbp *Cla*I-*Sal*I fragment from pTC900 into pTC400	This study
pTC2000	LT-I A, LT-IIb B	1.3-kbp *Cla*I-*Sal*I fragment from pTC900 into pTC700	This study
pTC3000	LT-IIa A, LT-I B	1-kbp *Bam*HI-*Eco*RV fragment from pTC304 into pTC801	This study
pTC4000	LT-IIb A, LT-I B	1.6-kbp *Cla*I-*Bam*HI fragment from pTC601 into pTC801	This study
Strains			
XL1-Blue	Nontoxinogenic	*recA1 supE44 relA1 lac* (F′ *proAB lacIq* Z M15 Tn*10*)	4
SA53	LT-IIa A, LT-IIa B	Parental strain expressing LT-IIa toxin	14
41	LT-IIb A, LT-IIb B	Parental strain expressing LT-IIb toxin	14
T1	LT-IIa A, LT-IIa B	XL1-Blue containing pTC304 and pTC400	This study
T2	LT-IIb A, LT-IIb B	XL1-Blue containing pTC601 and pTC700	This study
T3	LT-IIa A, LT-IIb B	XL1-Blue containing pTC304 and pTC700	This study
T4	LT-IIb A, LT-IIa B	XL1-Blue containing pTC601 and pTC400	This study
T5	LT-I A, LT-I B	XL1-Blue containing pTC900 and pTC801	This study
T6	LT-I A, LT-IIa B	XL1-Blue containing pTC900 and pTC400	This study
T7	LT-I A, LT-IIb B	XL1-Blue containing pTC900 and pTC700	This study
T8	LT-IIa A, LT-I B	XL1-Blue containing pTC304 and pTC801	This study
T9	LT-IIb A, LT-I B	XL1-Blue containing pTC601 and pTC801	This study

pentamers in vivo or in vitro, either in the presence or absence of mature homologous A polypeptides, but interaction with homologous A polypeptides increases the rate of association of B polypeptides and favors formation of holotoxin instead of B pentamers (16). Assembled B pentamers of type I enterotoxins do not associate with A subunits to form holotoxin (16). Hybrids between CT and LT-I can be produced by denaturing and renaturing mixtures of their A and B polypeptides in vitro, and such hybrid type I enterotoxins have toxicity comparable to that of native CT or LT-I (16, 44).

In the current study, we cloned the genes encoding the A and B polypeptides of LTp-I, LT-IIa, and LT-IIb and tested all possible pairwise combinations of their A and B genes, either in compatible plasmid vectors or in hybrid operon constructs, for complementation in *E. coli*. Our goals were to determine whether heterologous A and B polypeptides from type I and type II enterotoxins can associate to form hybrid holotoxins and, if so, to characterize the hybrid holotoxins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The LTp-I-producing plasmid pEWD299 (8) and the plasmids pCP3727 expressing LT-IIa (37, 39) and pCP4185 expressing LT-IIb (38) have been described previously. Genes encoding individual toxin polypeptides were cloned into the vectors pBluescript KS+ (or KS−) (Stratagene) and pACYC184 (5) by using standard methods (30). Genes cloned into pBluescript vectors were oriented for expression under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter. *E. coli* XL1-Blue (Stratagene) was used as the host strain for all recombinants (4). Details of construction and phenotypes of the recombinant plasmids and strains used in this study are summarized in Table 1.

DNA procedures. DNA was isolated by using the Ish-Horowicz and Burke modification (24) of the alkaline extraction procedure of Birnboim and Doly (3). Agarose gel electrophoresis was performed by standard procedures (30), and restriction fragments used for cloning were isolated from gel slices by electroelution. Restriction enzymes and other DNA-modifying enzymes were used according to suppliers' specifications (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; and New England Biolabs, Beverly, Mass.). Transformations were performed by using $CaCl_2$-treated cells as described by Maniatis et al. (30).

Preparation of toxin-containing extracts. *E. coli* strains expressing cloned genes for toxin A and/or B polypeptides from recombinant plasmids were cultured in LB broth (42). Ampicillin (75 μg/ml) and/or chloramphenicol (25 μg/ml) was

dded as appropriate to select for plasmid-determined resistance traits. Cultures were incubated at 37°C with vigorous shaking. IPTG was added (0.5 to 1.0 μg/ml) at mid-log stage (optical density at 600 nm of ~0.2 to 0.4), and incubation was continued overnight. Cells were pelleted by centrifugation and resuspended in 1/30 of the original volume of phosphate-buffered saline (PBS). Periplasmic toxins or polypeptides were released either by sonication or by treatment with polymyxin B sulfate (2 mg/ml for 30 min at room temperature) (25). Sonicates and extracts were centrifuged to pellet debris, and supernatants containing toxin or subunits were collected and used as described below. Extracts were either assayed and used immediately or stored at 4°C and reassayed before use in other experiments.

Assays for biological activity of heat-labile enterotoxins. Methods to test for biological activity of the toxin by using Y1 adrenal cells have been described previously (29). Samples (100 μl) containing serial dilutions of toxin extracts in RPMI 1640 were applied to monolayers of Y1 adrenal cells and incubated for 16 h. One unit of activity was defined as the minimum dose of toxin required to give a 4+ reaction, corresponding to rounding of at least 75% of the cells in the monolayer (29), and biological activity (toxicity) was expressed as units per milliliter. Extracts from XL1-Blue cells harboring pBluescript and/or pACYC184 plasmids were used as controls and were not toxic for Y1 cells. Purified LTp-I, LT-IIa, and LT-IIb toxins were used as positive controls. It is not possible to convert toxic activity of extracts directly to enterotoxin concentration, because specific activity depends on the ratio of nicked toxin to unnicked toxin. For the purified, fully nicked toxins, one toxic unit corresponds to approximately 12.5 pg of LTp-I (21), 0.5 pg of LT-IIa (21), and 3 pg of LT-IIb (15).

Solid-phase radioimmunoassays. Ganglioside-dependent solid-phase radioimmunoassays with monoclonal antibodies (MAb) directed against the A polypeptides of heat-labile enterotoxins were used to measure the amounts of immunoreactive holotoxin in extracts (20). A solution containing gangliosides GM1, GD1a, and GD1b (Matreya, Inc., Pleasant Gap, Pa.) at 0.8 μg of each per ml in PBS was prepared, and 25 μl of the mixed ganglioside solution (20 ng of each ganglioside) was added to each well of a 96-well polyvinyl microtiter plate (Dynatech, Alexandria, Va.). The plates were incubated at room temperature overnight, and unbound gangliosides were removed by washing the plates with PBS. The wells were then blocked by treatment for 30 min at room temperature with PBS containing 10% horse serum (PBS-10; GIBCO). All subsequent steps were performed at 37°C. Extracts containing toxins were serially diluted by using PBS-10, and 25-μl samples of each dilution were applied to the wells and incubated for 3 h. Wells were then washed with PBS containing 1% horse serum (PBS-1) and probed with 25 μl of hybridoma cell culture supernatant containing the appropriate mouse MAb specific for the A subunit present in the extract: MAb 4B6 (3.6 μg/ml) for LTp-I, MAb 5C5 (3.8 μg/ml) for LT-IIa, and MAb 19G5 (concentration not determined) for LT-IIb. The isotypes of MAb 4B6 and MAb 5C5 are immunoglobulin G1 (IgG1); that of MAb 19G5 is IgA. Details concerning the preparation and characterization of MAb 5C5 and MAb 19G5 will be described elsewhere (18a). None of these MAb cross-reacts with any of the enterotoxin B subunits. After 1 h of incubation, unbound antibodies were removed by rinsing with PBS-1. Wells were then probed with a 1:2,000 dilution of rabbit anti-mouse IgG (Sigma, St. Louis, Mo.) or rabbit anti-mouse IgA (Sigma), as appropriate, for 1 h, washed with PBS-1, and incubated for 90 min with ^{125}I-labelled goat anti-rabbit IgG (Sigma). After being washed with PBS-1, wells were cut from the plate, and the radioactivity of the ^{125}I-labelled goat anti-rabbit IgG bound to the well was counted by using a Micromedic Model MEplus Gamma Counter. Known amounts of purified LTp-I, LT-IIa, and LT-IIb toxins were used as standards, and immunoreactive holotoxin was expressed as micrograms of the standard enterotoxin containing the same A subunit as the extract per milliliter.

A modification of the procedure reported by Fukuta et al. (11) was used to measure the relative binding activities of hybrid toxins for different gangliosides. Polyvinyl microtiter plates were prepared as above, except that each well contained only one of the three gangliosides (GM1, GD1a, or GD1b) and the ganglioside was serially diluted in PBS in successive wells in a twofold series from 25 ng to 12 pg per well. Extracts were diluted to 200 ng of immunoreactive holotoxin per ml, and each extract was assayed on each of the three ganglioside-containing plates. The plates were incubated for 3 h and processed as described above.

Immunoadsorption of wild-type and hybrid holotoxins. To confirm that the biological activity of extracts containing homologous and heterologous toxin subunits was associated with holotoxin, Sepharose beads conjugated with streptococcal G-protein (Pharmacia, Inc., Piscataway, N.J.) were used to make specific immunoadsorbents. Aliquots (100 μl) of Sepharose–G-protein beads were incubated overnight with hybridoma supernatants containing MAb specific for the B subunits present in the extract to be adsorbed: MAb 15G3 for the B subunit of LT-IIa (63 μg of IgG per ml), MAb 20C9 for the B subunit of LT-IIb (24 μg of IgG per ml), and MAb 12G5 for the B subunit of LTp-I (70 μg of IgG per ml) (2). All three MAb are IgG1 isotypes. Details concerning the preparation and characterization of MAb 15G3 and MAb 20C9 will be described elsewhere (18a). None of these B-polypeptide-specific MAb cross-reacts with any of the toxin A polypeptides. After incubation, the beads were washed extensively to remove unbound MAb, and the beads were then used to adsorb 220 μl of toxin extracts which had been previously diluted to between 40 and 640 U/ml of biologically-active holotoxin. The titers of adsorbed and unadsorbed extracts were determined to ascertain the smallest dose of each that was cytotoxic in the Y1 adrenal cell assay. Solutions of purified LTp-I (873 pg/ml), LT-IIa (68 pg/ml), and LT-IIb (2.6 ng/ml) toxins were used as controls.

In vitro assembly of holotoxin from A and B subunits. To determine whether A and B subunits could assemble in vitro to form holotoxin, extracts from strains expressing the A or B subunit of LTp-I, LT-IIa, or LT-IIb were mixed in all pairwise combinations of A and B subunits. Equal volumes (10 to 50 μl) of A and B subunit extracts were combined, and the heterologous and homologous mixtures were incubated for 10 min at 37°C. Mixtures were then applied to microtiter plates sensitized with a mixture of GM1, GD1a, and GD1b gangliosides (20 ng of each per well) and measured for immunoreactive holotoxin by solid-phase radioimmunoassay using A-polypeptide-specific MAb, as described above. Samples containing only A or B subunit extracts were used as controls, and purified LTp-I, LT-IIa, and LT-IIb toxins were used as standards. To determine the effect, if any, of polymyxin B sulfate on assembly, sonicated extracts were also assayed. Polymyxin extracts and sonicated extracts behaved similarly in in vitro assembly experiments (data not shown). Similar mixtures of extracts containing A and B subunits were also measured by Y1 adrenal cell assays to determine whether cytotoxic holotoxin was produced. Con-

TABLE 2. Immunoreactivity and biological activity of heat-labile enterotoxins with homologous and heterologous A and B subunits encoded by structural genes on separate plasmids or in hybrid operons

Toxin composition		Activity of toxin subunits encoded by genes on:					
		Complementary plasmid			Operon fusion		
Subunit A	Subunit B	Biological activity[a] (U/ml)	Toxin antigen[b] (μg/ml)	Sp act (U/μg)	Biological activity (U/ml)	Toxin antigen (μg/ml)	Sp act (U/μg)
LT-IIa	LT-IIa	27,000	5.1	5,270	656,000	80.0	8,200
LT-IIb	LT-IIb	32,000	87.5	370	23,300	13.6	1,790
LT-I	LT-I	20,480	2.5	8,190	64,000	22.0	2,910
LT-IIa	LT-IIb	4,000	1.0	4,000	8,000	1.7	4,700
LT-IIb	LT-IIa	9,000	6.4	1,400	128,000	22.2	5,820
LT-I	LT-IIa	640	—[c]	—	640	—	—
LT-I	LT-IIb	320	—	—	1,280	—	—
LT-IIa	LT-I	1,000	1.0	1,000	640	0.2	2,600
LT-IIb	LT-I	640	2.1	302	1,280	2.5	512

[a] One unit of toxin is the smallest dose needed to produce a response in 75 to 100% of cells in a Y1 adrenal cell bioassay (29). Cells expressing A subunit alone or B subunit alone were not toxigenic.

[b] The amount of holotoxin in each extract was measured by solid-phase radioimmunoassay (11). Extracts were incubated in microtiter wells sensitized with 20 μg each of GM1, GD1a, and GD1b, and probed with A subunit-specific MAb 4B6 (LT-I) (2), MAb 5C5 (LT-IIa), or MAb 19G5 (LT-IIb). In each case, the immunoreactivity was expressed as the amount of holotoxin containing the same A subunit that produces an equivalent response in the radioimmunoassay.

[c] —, MAb specific to the A subunit failed to bind to holotoxin.

trol extracts from strains expressing only A or B polypeptides were not cytotoxic.

RESULTS

To facilitate complementation studies, DNA fragments that encode individual A or B polypeptides of LTp-I, LT-IIa, or LT-IIb were isolated and cloned into compatible plasmid vectors (Table 1). Each A gene with its native promoter was cloned into pACYC184 (Cm^r, P15A replicon), and each B gene without its native promoter was cloned into pBluescript KS+ (Amp^r, ColE1 replicon) under control of the IPTG-inducible *lac* promoter. Pairs of the compatible plasmids carrying each possible combination of cloned A and B genes were transformed into *E. coli* XL1-Blue, and cotransformants were selected and maintained by growth in the presence of ampicillin and chloramphenicol. To control for artifacts resulting from different plasmid copy numbers or expression of the A and B genes from promoters of different strength, hybrid operons containing each combination of A and B genes expressed under control of the *lac* promoter in pBluescript KS+ were also constructed, introduced into *E. coli* XL1-Blue, and maintained under ampicillin selection.

Strains expressing each pair of A and B polypeptides, either on separate plasmids or as hybrid operons, were cultured in LB broth until mid-log phase and induced overnight with IPTG. Periplasmic contents were released by treatment with polymyxin B, and the extracts were tested for toxicity in Y1 adrenal cell assays and for immunoreactive holotoxin in solid-phase radioimmunoassays (Table 2). Extracts from negative controls that contained only an A or a B polypeptide were not toxic and contained no immunoreactive holotoxin (data not shown). Extracts from positive controls that contained the A and B polypeptides of a single enterotoxin (either LTp-I, LT-IIa, or LT-IIb) were highly toxic and contained substantial amounts of immunoreactive holotoxin with high specific activity. These positive controls demonstrated that LTp-I, LT-IIa, and LT-IIb can all be produced as mature periplasmic holotoxins in *E. coli* by complementation between the cloned genes for their A and B polypeptides.

Extracts from all of the strains that expressed genes for heterologous A and B polypeptides, including those that produced a combination of type I and type II polypeptides, were also toxic for Y1 adrenal cells (Table 2). The total toxic activity of extracts containing the hybrid toxins was usually less than the toxicity of extracts containing the wild-type holotoxins. Immunoreactive hybrid holotoxins were detected in all extracts, except those containing the A polypeptide of LTp-I and the B polypeptide of either LT-IIa or LT-IIb. The hybrid holotoxins in these exceptional extracts were also undetectable as antigens when any of several MAb against the A subunit of LTh-I (1, 2) was substituted for MAb 4B6 in the solid-phase radioimmunoassay (data not shown). Possible explanations for failure to detect these specific hybrid toxins in ganglioside-dependent solid-phase radioimmunoassays include failure of the A polypeptide to fold in a manner that permits expression of some conformation-dependent epitopes, masking of some epitopes of the A polypeptide by abnormal folding or by association with the heterologous B polypeptide, or deficient ganglioside-binding activity of the hybrid holotoxin. The amounts of toxic activity and immunoreactive holotoxin produced by strains that expressed a hybrid enterotoxin operon under control of the *lac* promoter in pBluescript KS+ were usually greater than the amounts produced by strains that expressed the same A and B polypeptides from separate, compatible plasmids.

To confirm by an independent method that toxicity was due to formation of hybrid holotoxins, extracts containing each combination of A and B polypeptides were adsorbed with Sepharose–streptococcal G-protein beads coated with MAb against the B polypeptides that they contained (Table 3). None of the MAb against a specific B polypeptide cross-reacted with any of the A polypeptides or with the other B polypeptides (data not shown). Purified LTp-I, LT-IIa and LT-IIb toxins were used as positive controls, and Sepharose–streptococcal G-protein beads coated with MAb specific for the other B polypeptides served as negative controls for the absorption experiments. In each case, toxicity was removed by immunoabsorption only when the

TABLE 3. Removal of biological activity of heat-labile enterotoxins by adsorption with B subunit-specific MAb

Toxin Composition[a]		B subunit-specific MAb[b]	Biological activity removed (%)
Subunit A	Subunit B		
LT-IIa	LT-IIa	15G3	>88
LT-IIb	LT-IIb	20C9	>94
LT-I	LT-I	12G5	99
LT-IIa	LT-IIb	20C9	94
LT-IIb	LT-IIa	15G3	>99
LT-I	LT-IIa	15G3	88
LT-I	LT-IIb	20C9	0[c]
LT-IIa	LT-I	12G5	94
LT-IIb	LT-I	12G5	94
LT-IIa[d]	LT-IIa[d]	15G3	99
LT-IIb[d]	LT-IIb[d]	20C9	88
LT-I[d]	LT-I	12G5	97

[a] Toxin-containing bacterial extracts (unless otherwise noted) were prepared from strains expressing toxin from wild-type or hybrid operons.
[b] Specificities of MAb used: 15G3 (LT-IIa B subunit), 20C9 (LT-IIb B subunit), and 12G5 (LT-I B subunit) (2). The antibodies do not cross-react.
[c] MAb 20C9 does not bind the hybrid holotoxin.
[d] Purified holotoxins were used as positive controls.

Sepharose–streptococcal G-protein beads were coated with MAb against the B polypeptide that was present in the extract or purified enterotoxin. The only exception was the extract that contained the A polypeptide of LTp-I and the B polypeptide of LT-IIb, from which toxicity was not absorbed by using MAb 20C9 or an alternative MAb 11E5 that was also specific for the B polypeptide of LT-IIb (data not shown). The hybrid holotoxin that failed to react with MAb against the B polypeptides in the immunoabsorption experiments also failed to react with MAb against the A polypeptide in the solid-phase radioimmunoassays shown in Table 2. These results were confirmed in several experiments using independently derived constructs for expression of the LTp-I A polypeptide and the LT-IIb B polypeptide.

Previous studies reported that GM1 is the preferred ganglioside receptor for LT-I (13) and that LT-IIa and LT-IIb preferentially bind to the gangliosides GD1b and GD1a, respectively (11). To confirm that the B polypeptides determine ganglioside-binding specificity for both type I and type II heat-labile enterotoxins, extracts from strains used for the complementation tests were tested in solid-phase radioimmunoassays to determine the relative binding activities of the corresponding hybrid holotoxins for GM1, GD1a, and GD1b (Fig. 1). In each case, the B polypeptide of the hybrid holotoxin determined its ganglioside-binding specificity. Hybrid holotoxins containing A polypeptides of LTp-I and B polypeptides of LT-IIa or LT-IIb were not tested, because we were unable to detect them in ganglioside-binding solid-phase radioimmunoassays with anti-A MAb.

Finally, the ability of various combinations of A and B polypeptides to form holotoxins in vitro was examined (Table 4). Previous studies demonstrated that mixtures of denatured A and B polypeptides of type I enterotoxins in urea can renature into active holotoxin when the urea is slowly removed by dialysis (16, 44), but holotoxin is not formed when the A and B subunits are simply mixed together in aqueous solutions at neutral pH (44). We confirmed these findings for mixtures of A and B polypeptides from LTp-I. Unexpectedly, we found that toxic, immunoreactive holotoxins formed spontaneously in mixtures of extracts containing any combination of A and B polypeptides of LT-IIa and LT-IIb, without subjecting them to denaturation in urea and dialysis. Furthermore, type II holotoxins also formed when extracts containing A polypeptides were added to ganglioside-coated wells to which B polypeptides had previously been adsorbed. Hybrid holotoxins were not formed from any mixture that combined a type I A polypeptide with a type II B polypeptide, or vice versa. These data demonstrated that the requirements for assembly of type II enterotoxins differ significantly from those required for assembly of type I toxins.

DISCUSSION

The assembly of multimeric proteins such as heat-labile enterotoxins requires the interaction of complementary domains of the constituent subunits and is dependent on subunit conformation (34). Recently, the three-dimensional structure of LTp-I was solved by X-ray diffraction (43). The A2 domain of LTp-I is inserted into a pore formed at the center of the B pentamer, and the A2 domain interacts noncovalently with alpha helical domains of the B polypeptides to form holotoxin. Since LT-I and CT are highly homologous in these regions of their A and B polypeptides, it is not surprising that hybrid holotoxins composed of purified LT-I and CT subunits can form stable and biologically active molecules. Experiments presented in this paper demonstrate that hybrid holotoxins comprised of type I and II subunits also can be formed, although the A2 domains of type I and II enterotoxins show only moderate amino acid sequence homology and the B polypeptides of type I and II enterotoxins have little, if any, significant homology. The data suggest that type I and type II toxins have common structural features that are required for holotoxin assembly but are not readily apparent from their primary amino acid sequences.

Hybrid toxins were expressed from strains containing all

TABLE 4. In vitro formation of biologically active holotoxin in mixtures of extracts containing A and B subunits

Toxin composition[a]		Immunoreactivity (μg/ml)[b]	Cytotoxicity[c]
Subunit A	Subunit B		
LT-IIa		0	−
LT-IIb		0	−
LT-I		0	−
	LT-I	0	−
	LT-IIa	0	−
	LT-IIb	0	−
LT-IIa	LT-IIa	2.0	+
LT-IIb	LT-IIb	1.9	+
LT-IIa	LT-IIb	2.8	+
LT-IIb	LT-IIa	12.5	+
LT-I	LT-IIa	0	−
LT-I	LT-IIb	0	−
LT-IIa	LT-I	0	−
LT-IIb	LT-I	0	−

[a] Equal volumes (10 to 50 μl) of polymyxin B sulfate extracts from strains expressing LT-II A or B subunits were mixed and incubated for 10 min at 37°C prior to the solid-phase radioimmunoassay assay for holotoxin or the Y1 adrenal cell assay for cytotoxicity (29).
[b] Specificities of the MAb used in the solid-phase radioimmunoassay: MAb 4B6 (LT-I) (2), MAb 5C5 (LT-IIa), and MAb 19G5 (LT-IIb).
[c] The mixture caused either a +4 rounding response (+) or no rounding (−) in a Y1 adrenal cell cytotoxicity assay.

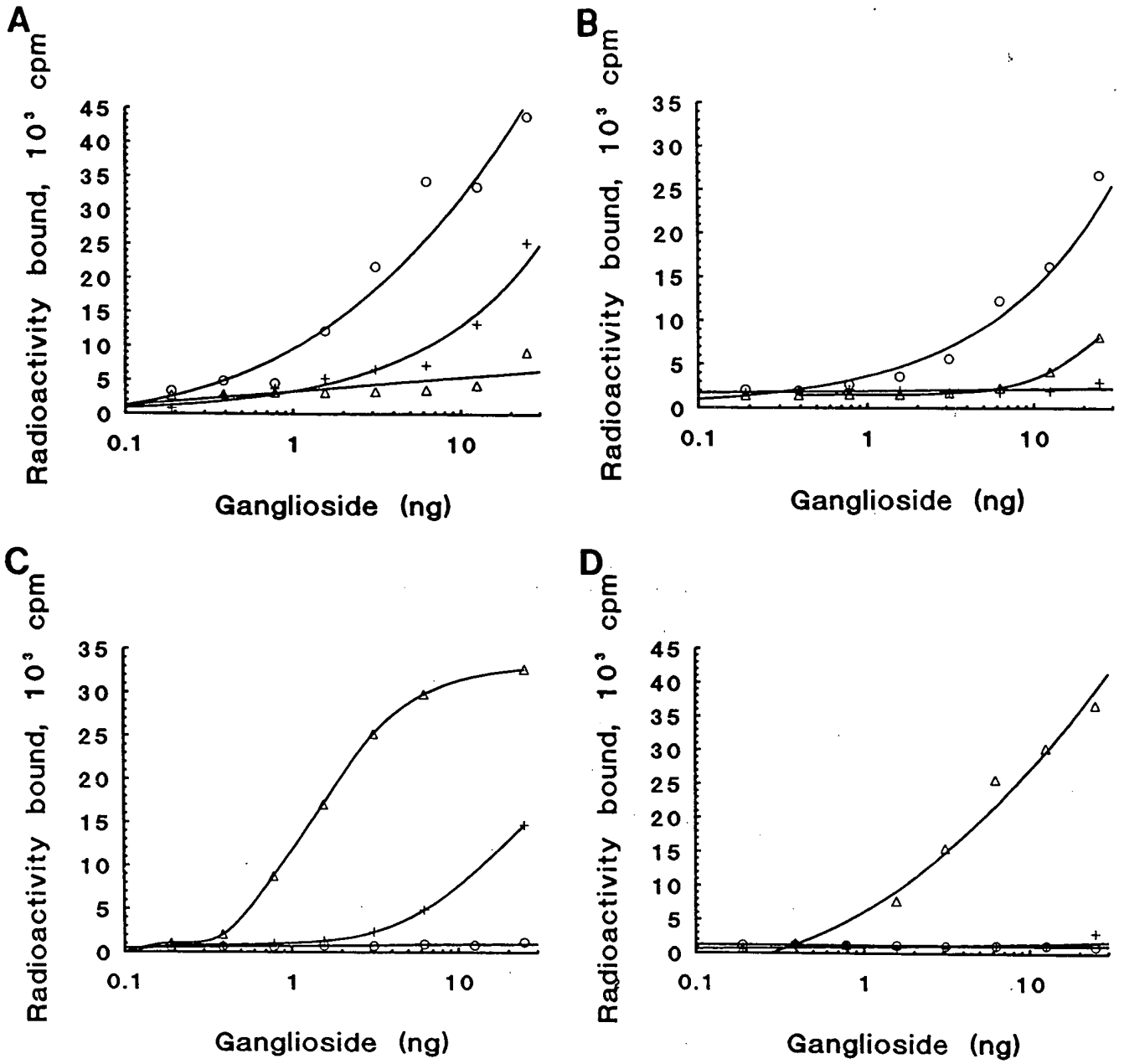
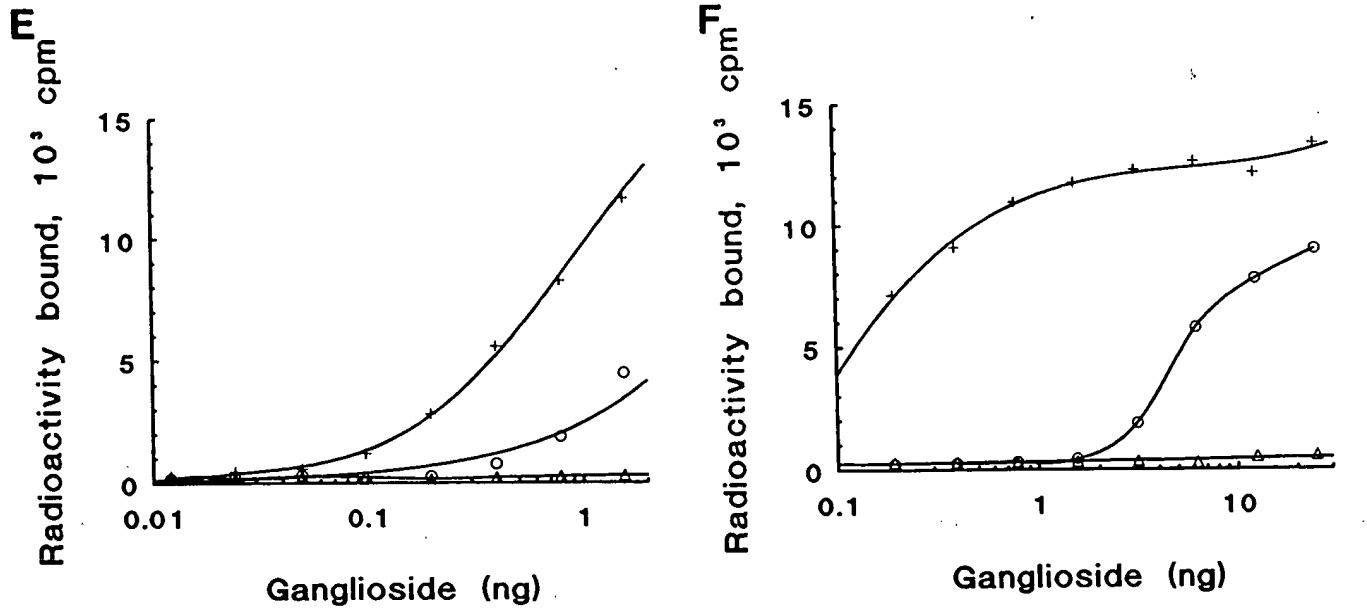


FIG. 1. Relative ganglioside-binding activities of toxins formed from homologous and heterologous combinations of A and B subunits from *E. coli* type I and type II enterotoxins. (A) Both A and B polypeptides from LT-IIa; (B) A polypeptide from LT-IIb and B polypeptide from LT-IIa; (C) both A and B polypeptides from LT-IIb; (D) A polypeptide from LT-IIa and B polypeptide from LT-IIb; (E) both A and B polypeptides from LTp-I; (F) A polypeptide from LT-IIa and B polypeptide from LTp-I; (G) A polypeptide from LT-IIb and B polypeptide from LTp-I. +, GM1; △, GD1a; ○, GD1b.

pairwise combinations of A and B genes from type I and type II enterotoxins on complementary plasmids or in operon fusions. The hybrid enterotoxins were usually detected in smaller amounts than the wild-type toxins containing A and B subunits, both in strains with complementing plasmids and in strains expressing both toxin subunits from a single operon fusion. Previous investigations have shown that prior treatment with trypsin was necessary to obtain the fully nicked form of purified LT-IIb toxin and to demonstrate its maximal toxicity (15). Attempts by Western blotting (immunoblotting) to determine the degree of nicking in these hybrid toxins were inconclusive, and treatment of the crude extracts with trypsin did not increase their toxicity (data not shown).

Extracts containing LTp-I A polypeptides and either LT-IIa or LT-IIb B polypeptides were slightly toxic on Y1 cells, but immunoreactive toxin was not demonstrated with any MAb among several that were specific for the A subunit of LTp-I or the B subunit of LT-IIa or LT-IIb. It is possible that specific epitopes on these particular hybrid toxins are conformationally altered or obscured. Alternatively, these particular hybrid toxins may be highly toxic but expressed at levels that are below the limit of sensitivity of the solid-phase



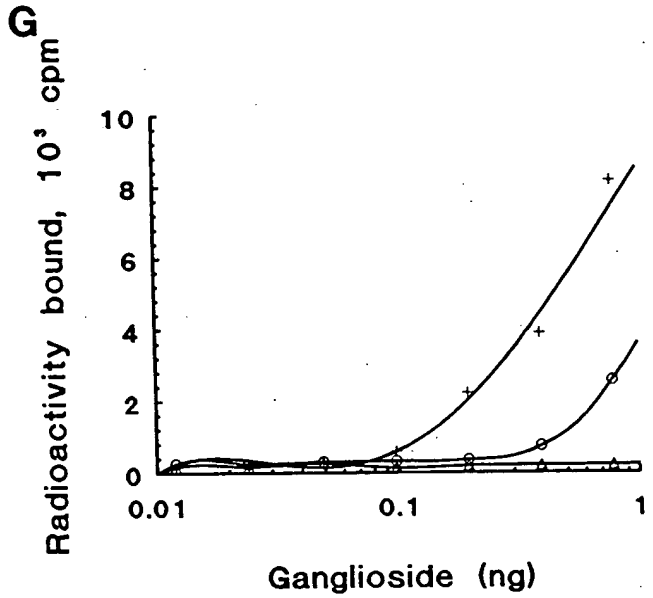


FIG. 1—*Continued.*

radioimmunoassays used in our experiments (approximately 100 pg per assay).

To demonstrate that the B polypeptides of type II enterotoxins determine their ganglioside-binding activity and to investigate the effects, if any, of different A polypeptides in modifying the ganglioside-binding activity or specificity of the B polypeptides in hybrid holotoxins, we studied the ganglioside-binding activities of all of the wild-type and hybrid holotoxins that we prepared. Our results demonstrated that each of the hybrid toxins maintained the same preference for ganglioside-binding that was exhibited by the native holotoxin with the same B subunit. We conclude that the B subunits alone determine ganglioside-binding specificities for both LT-I and LT-II enterotoxins.

Assembly of type I enterotoxins is thought to occur in a stepwise manner in vivo. The A polypeptides of type I enterotoxins do not assemble with mature B pentamers either in vivo or in vitro; instead, they appear to interact with an intermediate complex of B polypeptides (B_n, where n is less than 5) (18). Assembly of type I holotoxins does occur in vitro when A polypeptides and monomeric B polypeptides are denatured in acidic urea and slowly renatured by dialysis against appropriate buffers (16, 44). In contrast, the experiments reported here demonstrated that either homologous or heterologous mixtures of A and B polypeptides of LT-IIa and LT-IIb assembled readily in vitro, without prior exposure to denaturing conditions, to form immunoreactive and biologically active holotoxins. Further experiments will be needed to investigate the kinetics and stoichiometry for assembly of purified subunits of type II enterotoxins in vitro and compare the pathways for assembly of type II enterotoxins in vivo and in vitro.

In summary, all homologous and heterologous combinations of A and B polypeptides from type I and type II heat-labile enterotoxins are capable of assembling into active holotoxins in vivo, although not with equal efficiency. The data suggest that certain structural features necessary for interaction of A and B polypeptides during holotoxin assembly have been conserved among type I and type II enterotoxins, but these features are not evident from primary amino acid sequences. Determination of the three-dimensional structure of a representative type II enterotoxin is an important goal and will permit direct comparisons of the interactions between the A and B polypeptides in mature type I and type II heat-labile enterotoxins.

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US00603[illegible]4A

United States Patent [19]

Russell et al.

[11] **Patent Number: 6,030,624**

[45] **Date of Patent: Feb. 29, 2000**

[54] **MUCOSAL IMMUNOGENS FOR NOVEL VACCINES**

[75] Inventors: **Michael William Russell**; **Georgios Hajishengallis**; **Susan K. Hollingshead**, all of Birmingham; **Hong-Yin Wu**, Hoover; **Suzanne Mary Michalek**, Birmingham, all of Ala.

[73] Assignee: **UAB Research Foundation**, Birmingham, Ala.

[21] Appl. No.: **08/912,180**

[22] Filed: **Aug. 15, 1997**

Related U.S. Application Data

[60] Provisional application No. 60/024,074, Aug. 16, 1996, abandoned.

[51] **Int. Cl.**[7] **A61K 39/02**; A61K 39/09; C12N 1/21

[52] **U.S. Cl.** **424/200.1**; 424/93.2; 424/244.1; 435/252.3; 435/252.8

[58] **Field of Search** 435/320.1, 252.3, 435/252.33, 252.8; 530/69.3, 350, 403; 424/192.1, 197.11, 200.1, 236.1, 244.1, 261.1, 93.2

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Hajishengallis et al. Journal of Immunology 154 (9): 4322–32, May 1, 1995.

Redman et al. Infection and Immunity 63 (5): 2004–2011, May 1995.

Primary Examiner—Mary E. Mosher

Attorney, Agent, or Firm—Benjamin Aaron Adler

[57] **ABSTRACT**

The present invention provides chimeric proteins such as Salivary Binding Protein (SBR) coupled to the B subunit of cholera toxin. Such a chimeric protein, when expressed in attenuated *Salmonella typhymurium* produces significant increases in serum IgG and salivary IgA antibody levels after oral immunization. In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin A2/B chimeric protein expressed in *E. coli*. Intragastric immunization of SBR coupled to CTB in this chimeric protein form leads to increased antigen responsive T cells. In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin$^{\Delta A1}$ chimeric protein expressed in *Salmonella typhimurium*. Oral immunization using this recombinant plasmid results in increased serum IgG responses to antigen. Oral immunization using this recombinant plasmid also resulted in increased salivary IgA antibody responses to antigen.

11 Claims, 26 Drawing Sheets

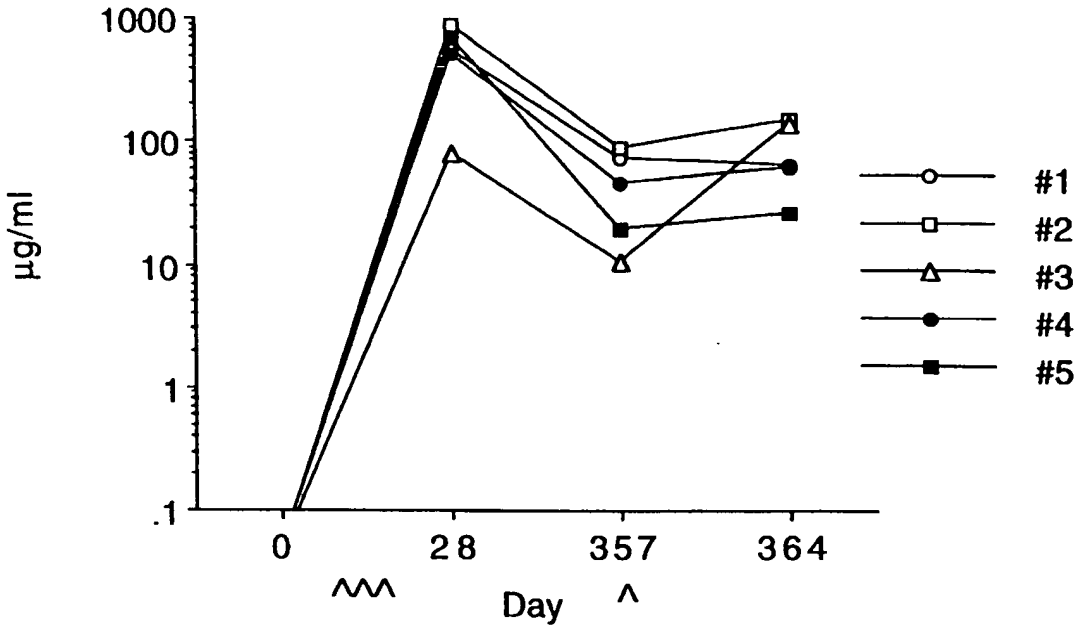
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 1A

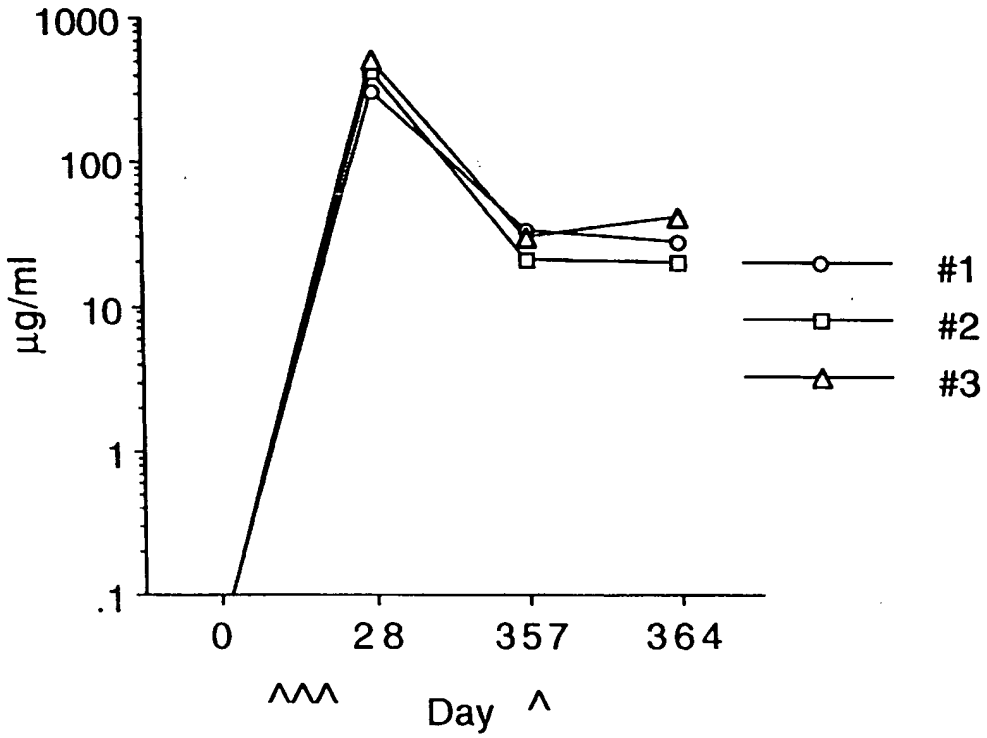
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3

Fig. 1B

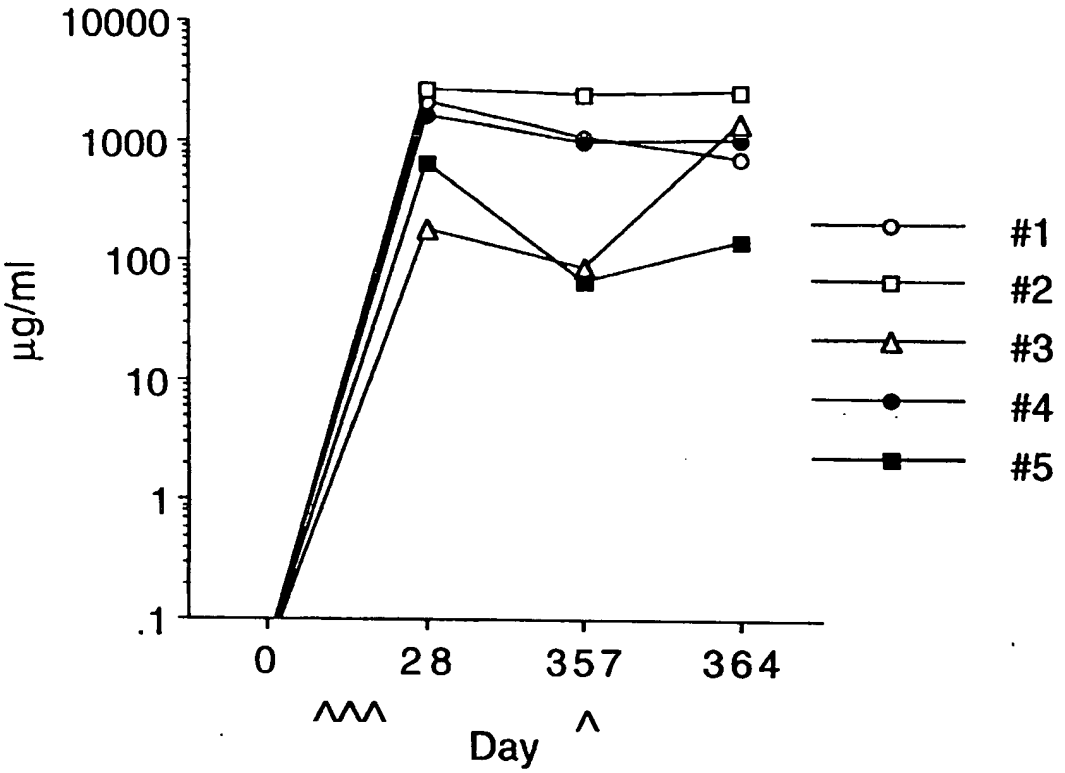
10000
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 1C

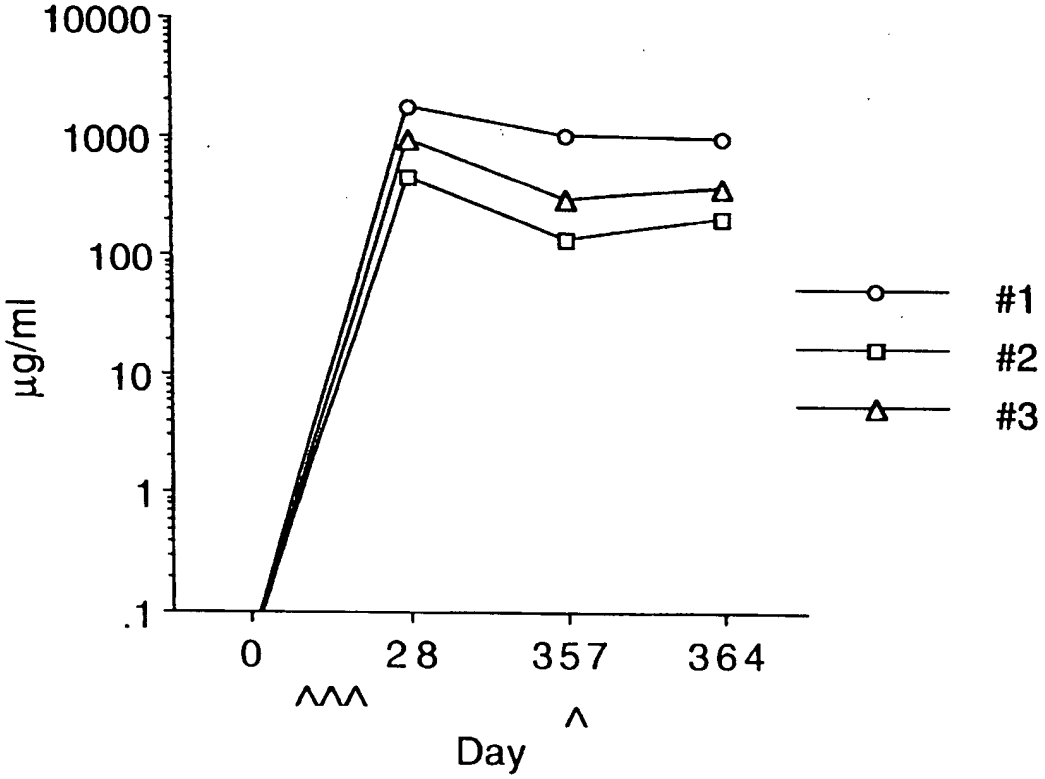
10000
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3

Fig. 1D

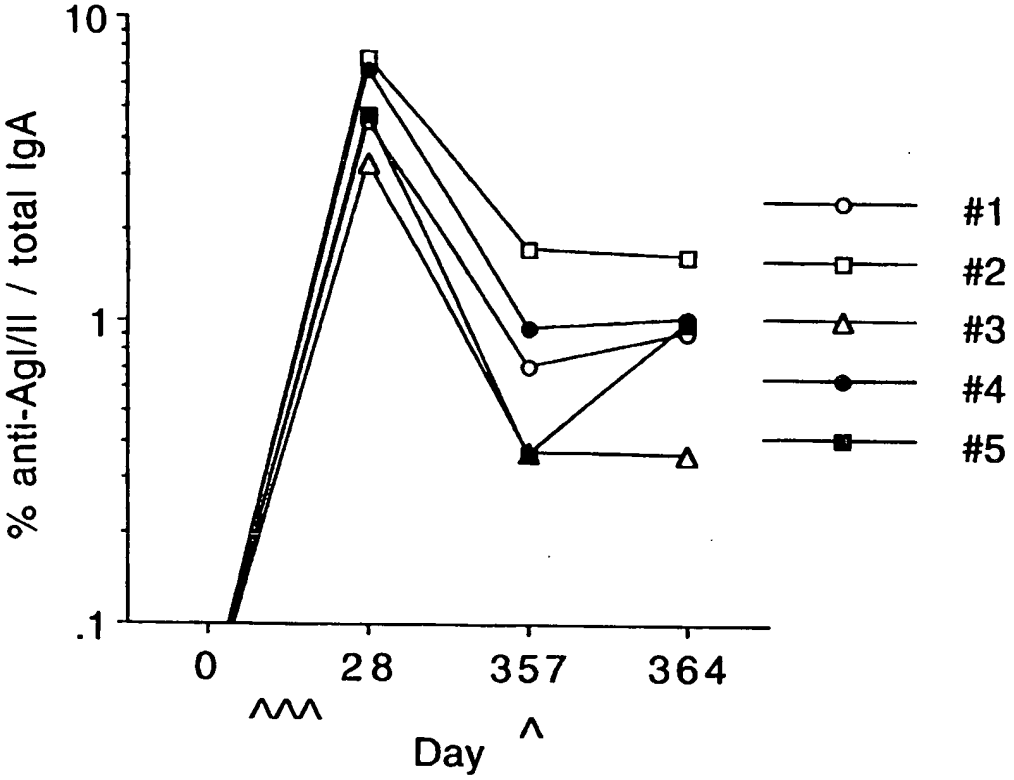
10
1
.1
% anti-AgI/II / total IgA
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 2A

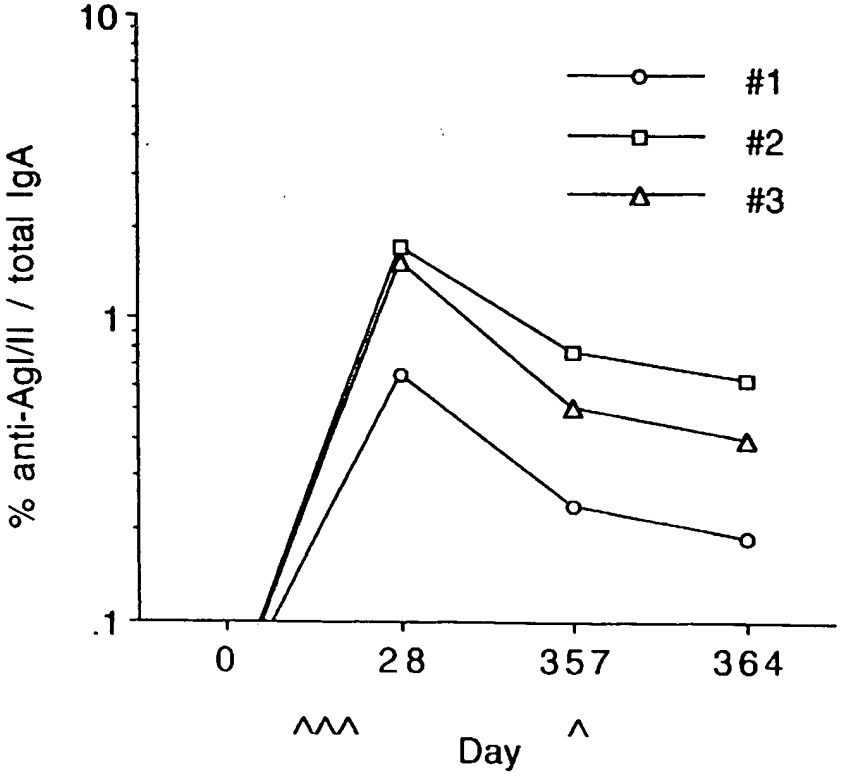
10
1
.1
% anti-AgI/II / total IgA
0
28
357
364
Day
#1
#2
#3

Fig. 2B

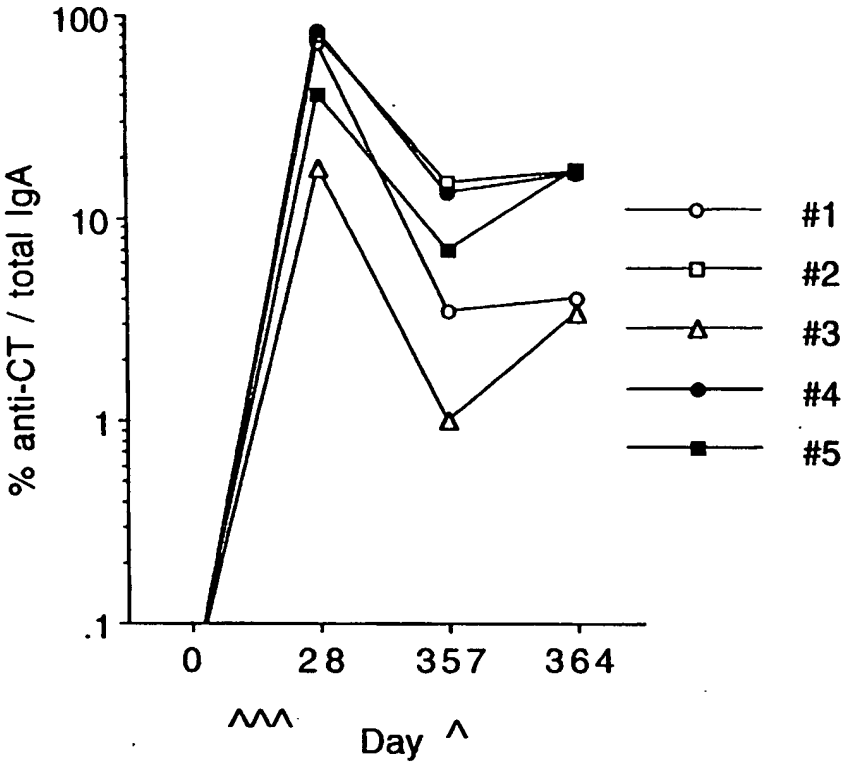
100
10
1
.1
% anti-CT / total IgA
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 2C

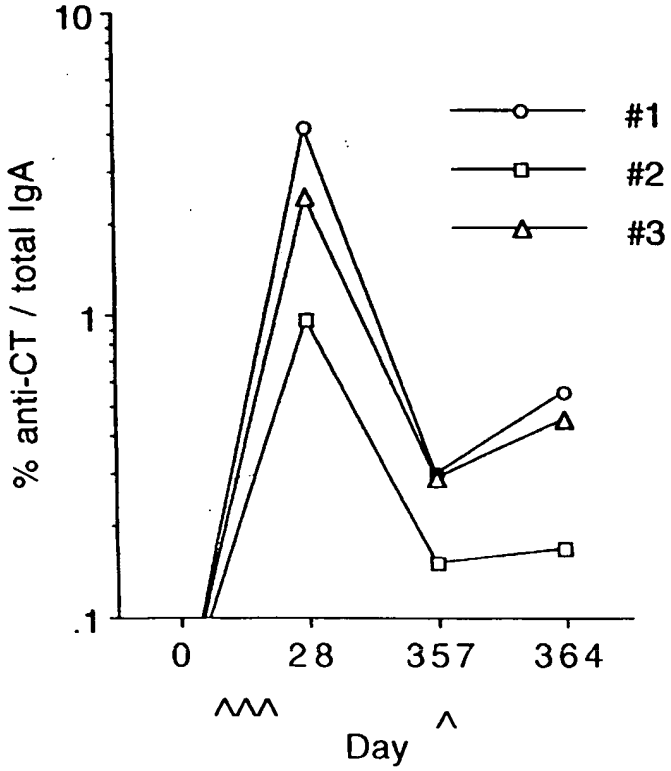
10
1
.1
% anti-CT / total IgA
0
28
357
364
Day
#1
#2
#3

Fig. 2D

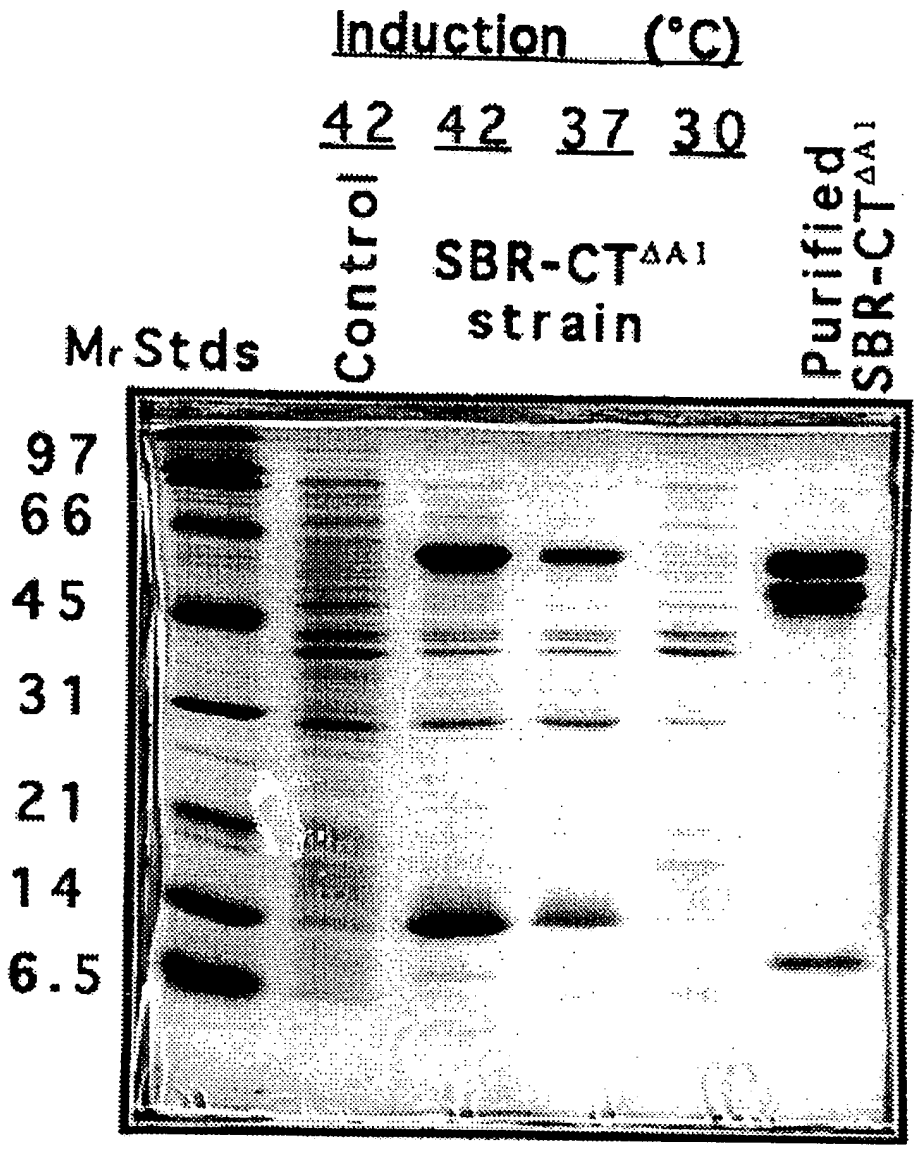
Induction (°C)
42
42
37
30
Control
SBR-CT$^{\Delta A1}$ strain
Purified SBR-CT$^{\Delta A1}$
Mr Stds
97
66
45
31
21
14
6.5

FIG. 3

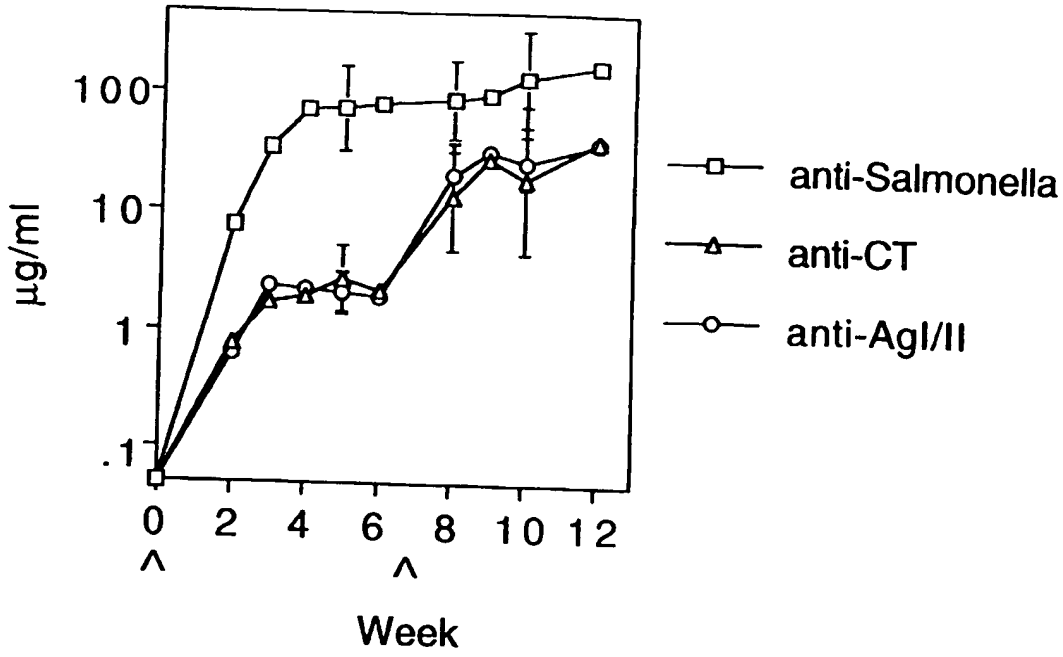
100
10
1
.1
µg/ml
0
2
4
6
8
10
12
Week
anti-Salmonella
anti-CT
anti-AgI/II

Fig. 4A

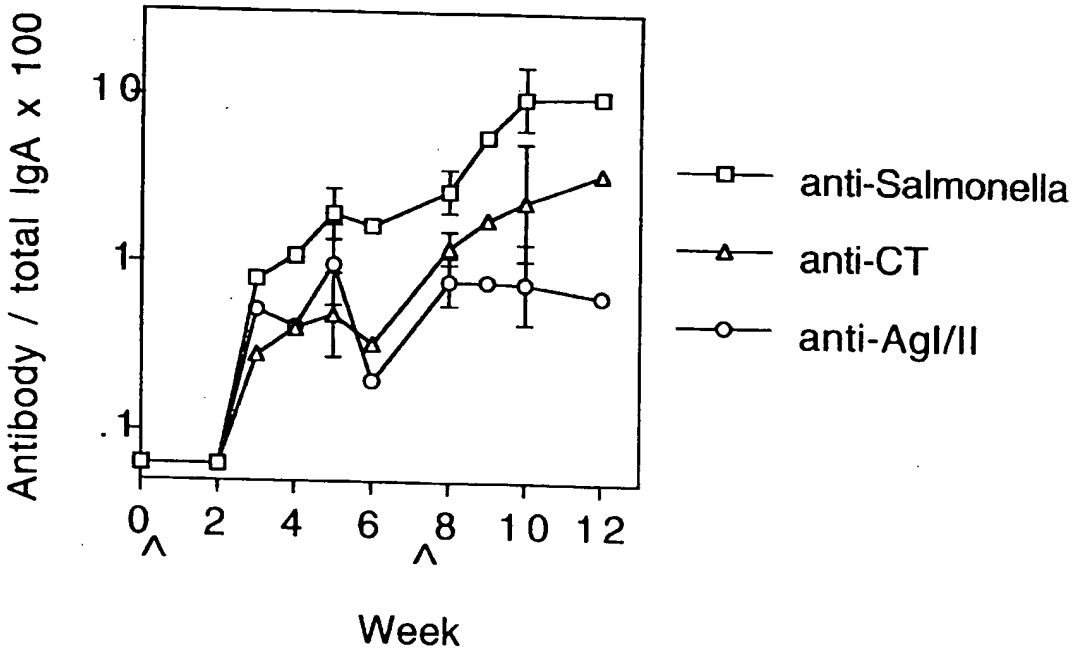
10
1
.1
Antibody / total IgA x 100
0
2
4
6
8
10
12
Week
anti-Salmonella
anti-CT
anti-AgI/II

Fig. 4B

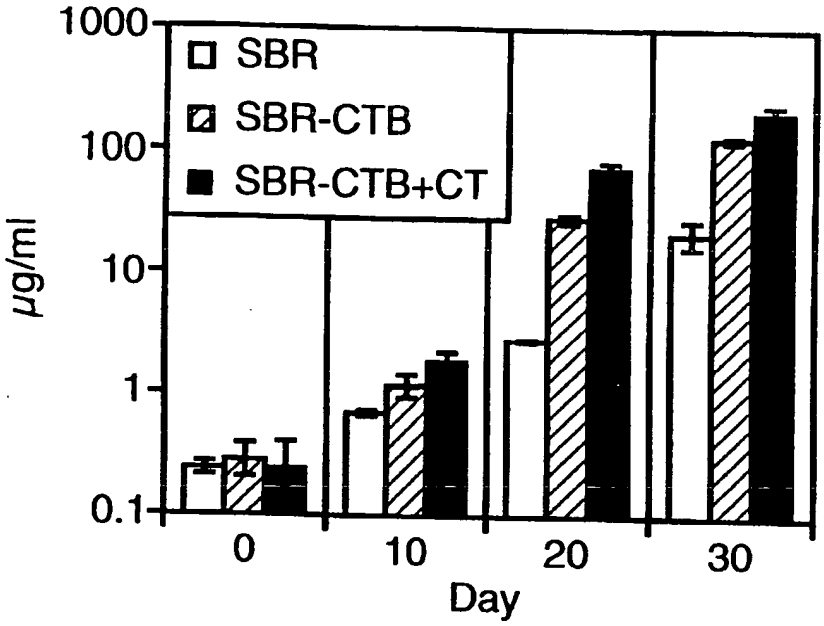
1000
100
10
1
0.1
μg/ml
SBR
SBR-CTB
SBR-CTB+CT
0
10
20
30
Day

Fig. 5A

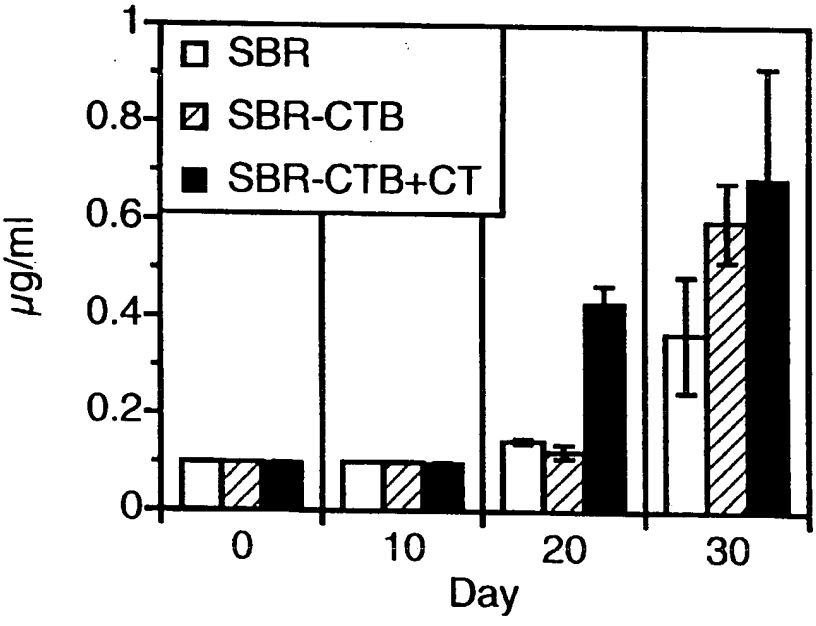
1
0.8
0.6
0.4
0.2
0
μg/ml
SBR
SBR-CTB
SBR-CTB+CT
0
10
20
30
Day

Fig. 5B

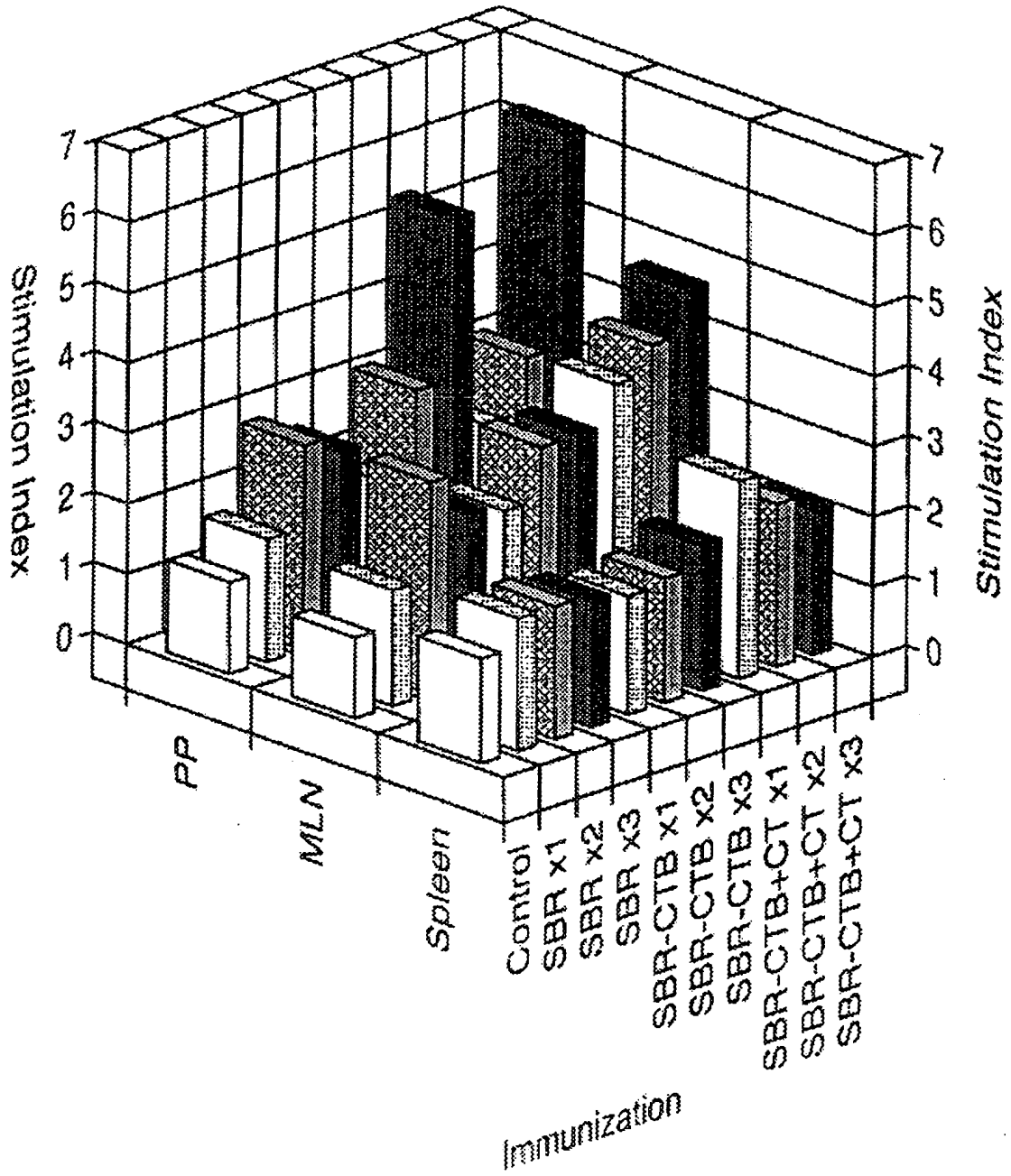
Stimulation Index
0
1
2
3
4
5
6
7
PP
MLN
Spleen
Control
SBR x1
SBR x2
SBR x3
SBR-CTB x1
SBR-CTB x2
SBR-CTB x3
SBR-CTB+CT x1
SBR-CTB+CT x2
SBR-CTB+CT x3
Immunization
Stimulation Index

Fig. 6

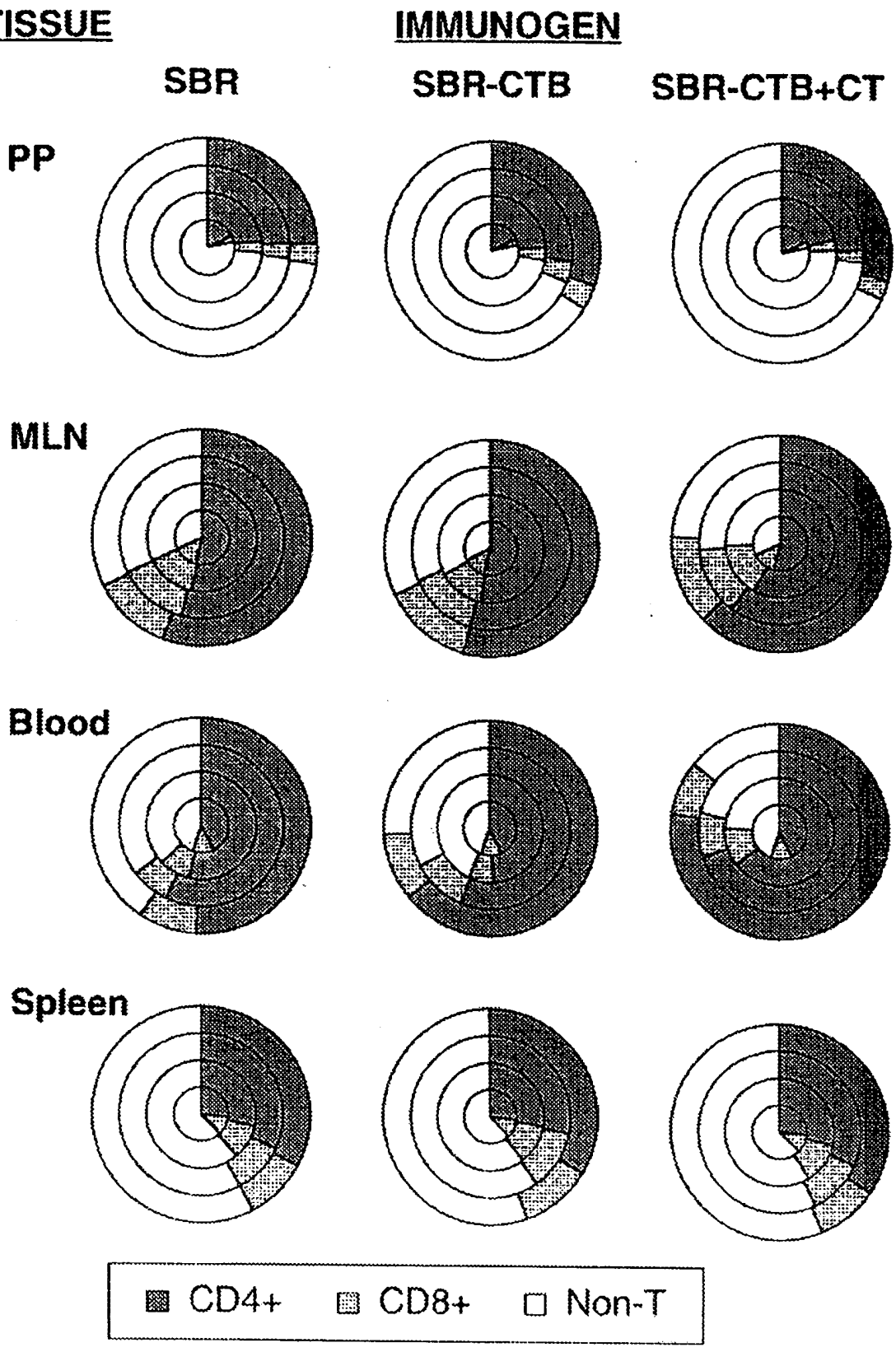
TISSUE
IMMUNOGEN
SBR
SBR-CTB
SBR-CTB+CT
PP
MLN
Blood
Spleen
CD4+
CD8+
Non-T

Fig. 7

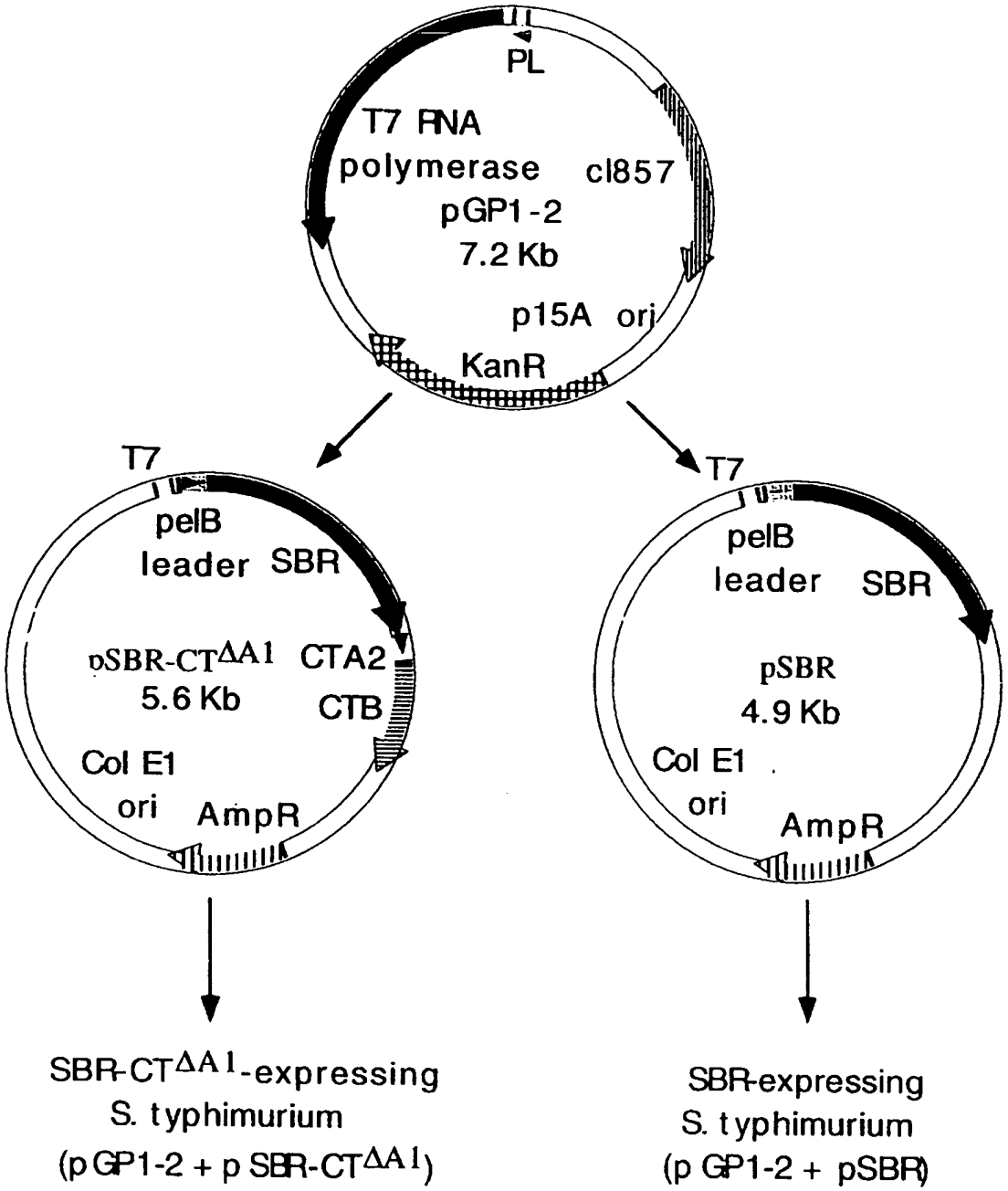
PL
T7 RNA
polymerase
cI857
pGP1-2
7.2 Kb
p15A ori
KanR
T7
pelB
leader
SBR
pSBR-CTΔA1
5.6 Kb
CTA2
CTB
Col E1
ori
AmpR
T7
pelB
leader
SBR
pSBR
4.9 Kb
Col E1
ori
AmpR
SBR-CTΔA1-expressing
S. typhimurium
(pGP1-2 + pSBR-CTΔA1)
SBR-expressing
S. typhimurium
(pGP1-2 + pSBR)

Fig. 8A

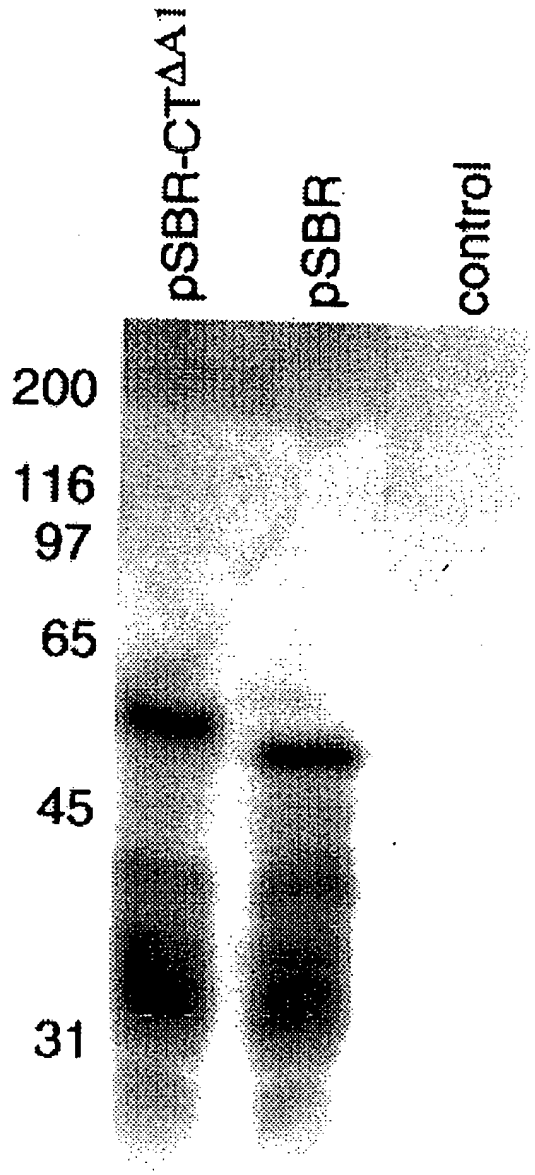

FIG. 8B

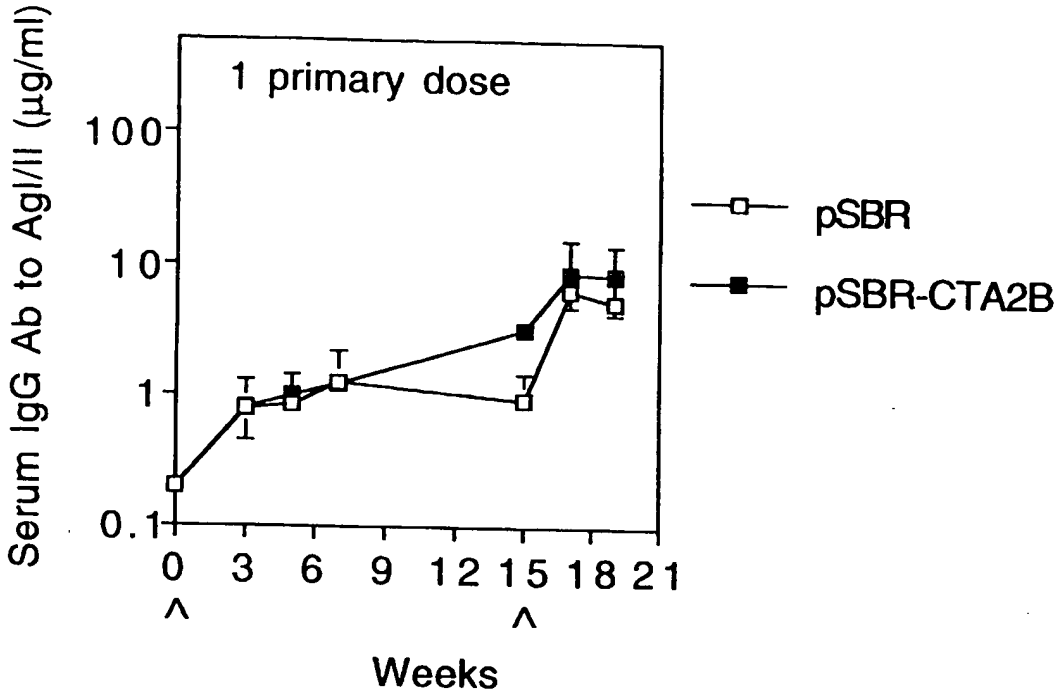
1 primary dose
Serum IgG Ab to AgI/II (µg/ml)
100
10
1
0.1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2B

Fig. 9A

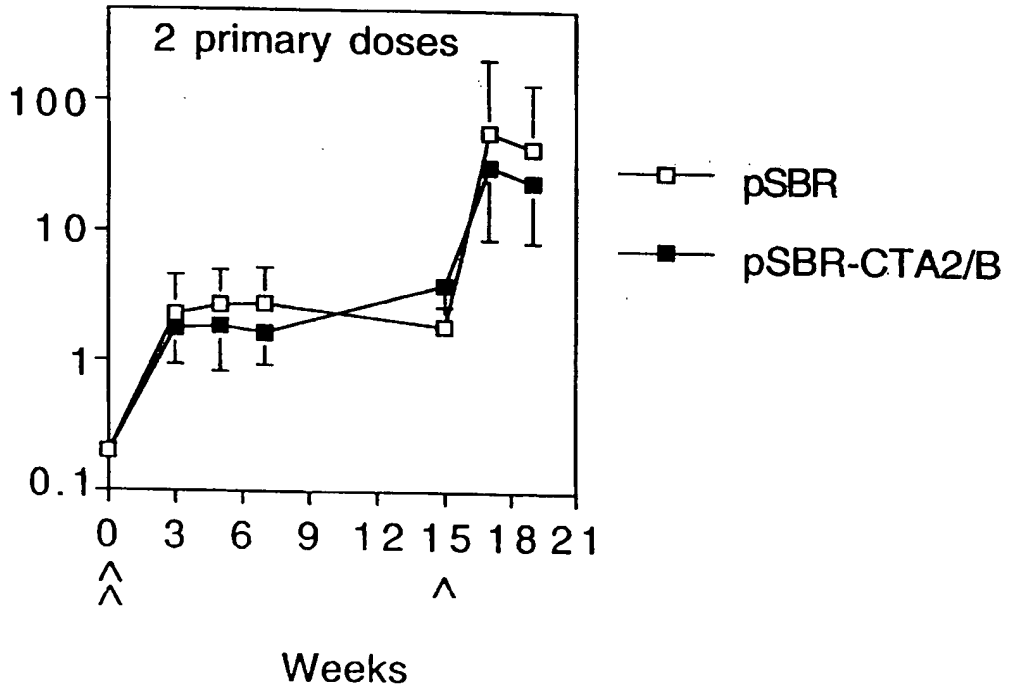
2 primary doses
100
10
1
0.1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2/B

Fig. 9B

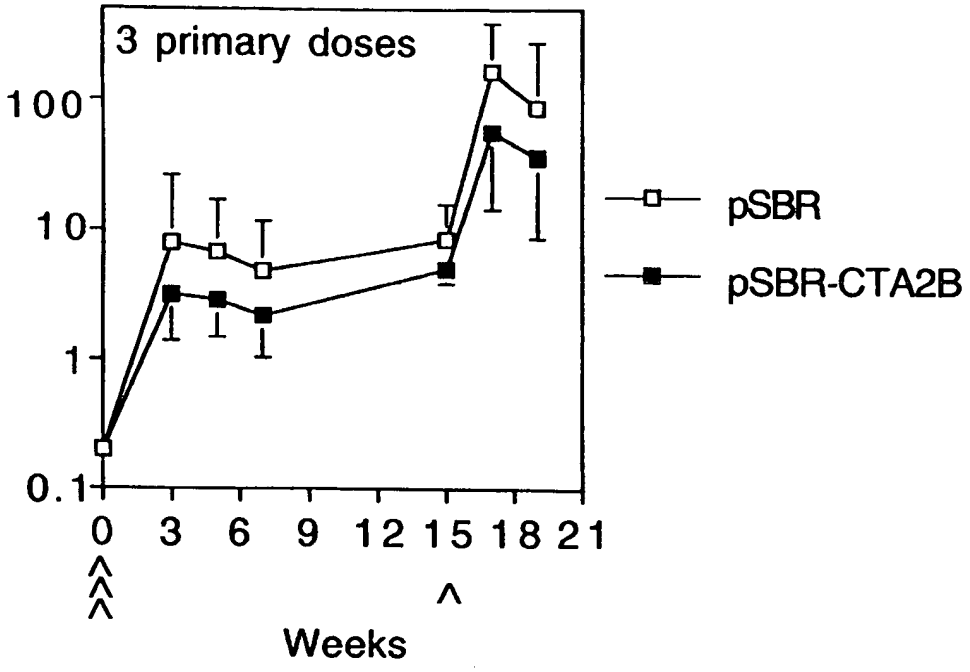

Fig. 9C

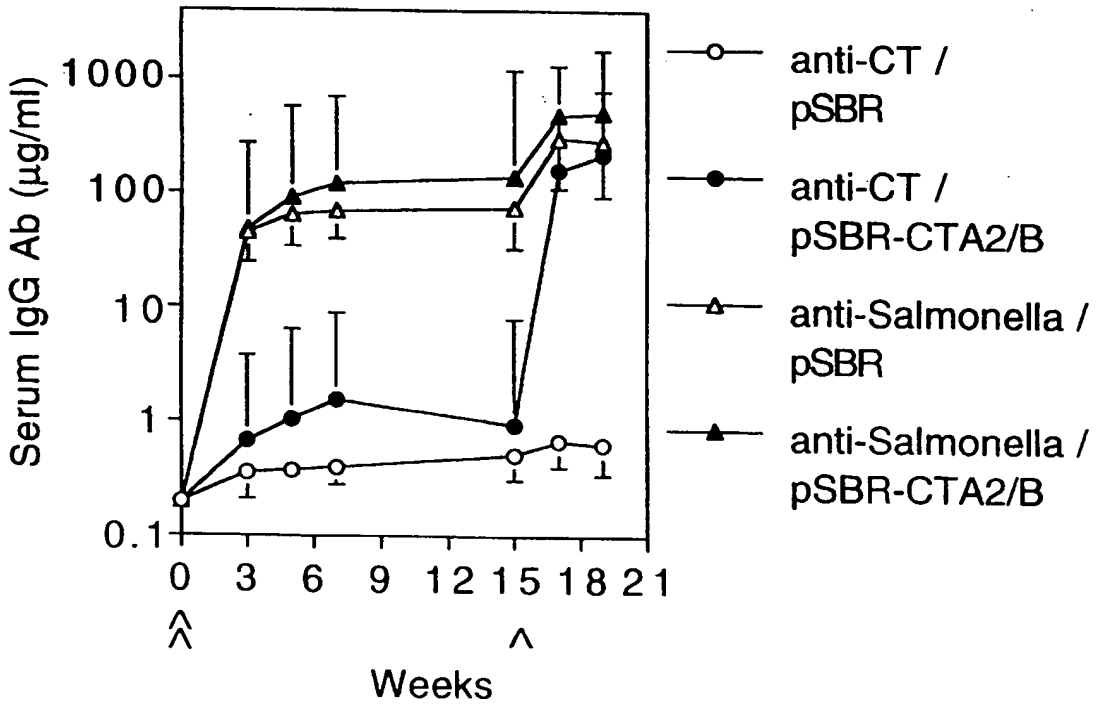

Fig. 10A

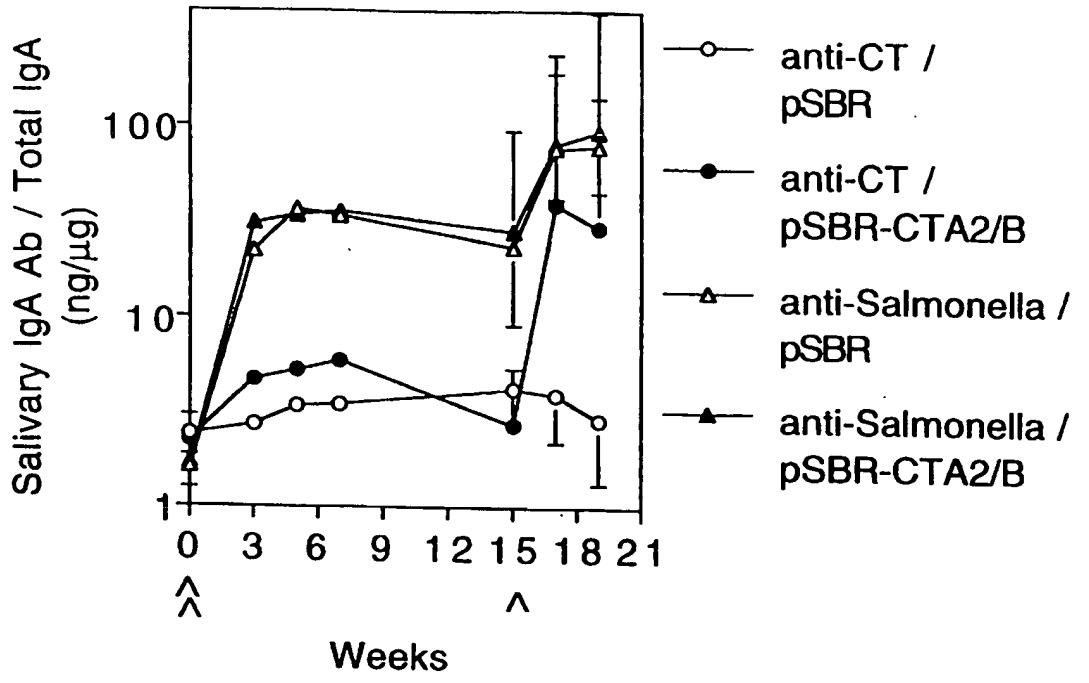

Fig. 10B

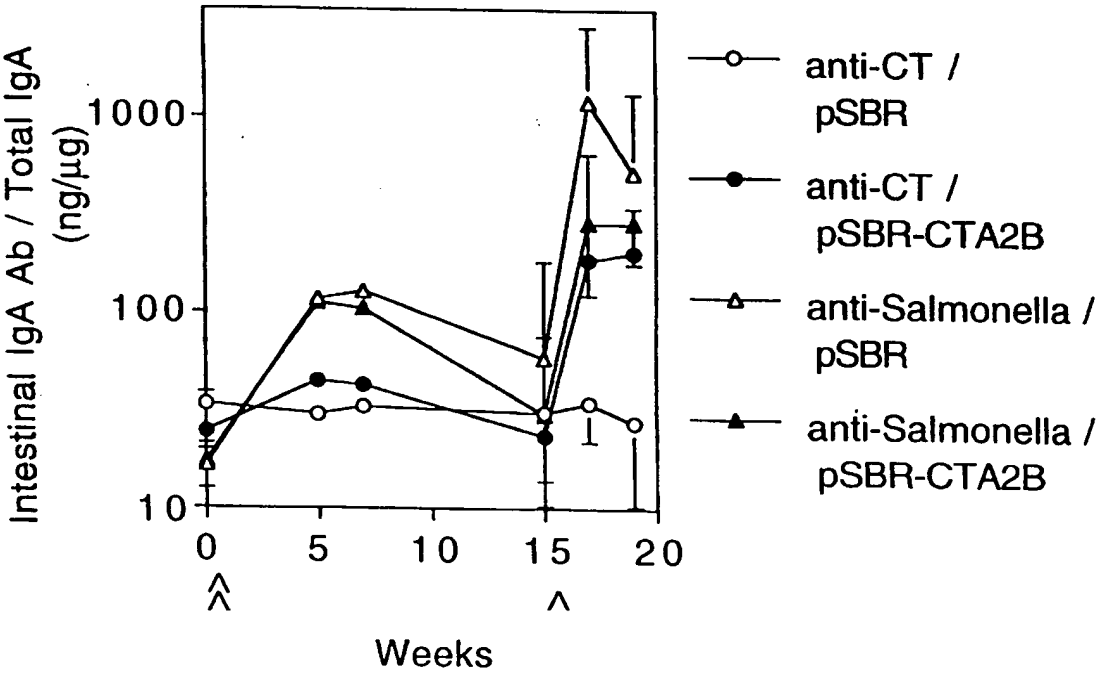

Fig. 10C

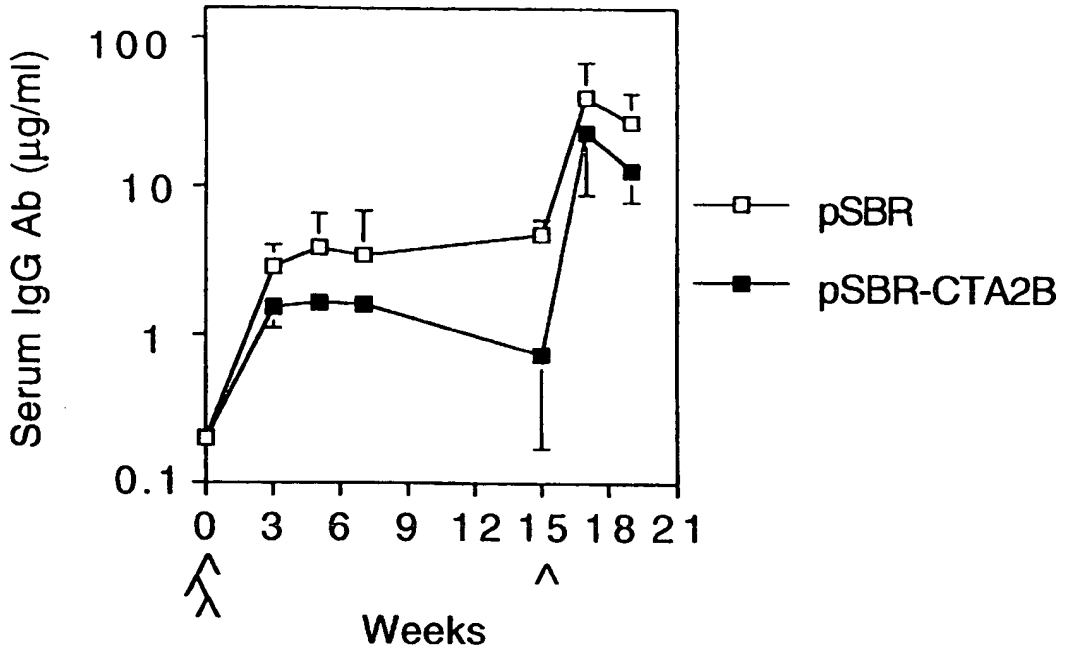

Fig. 11A

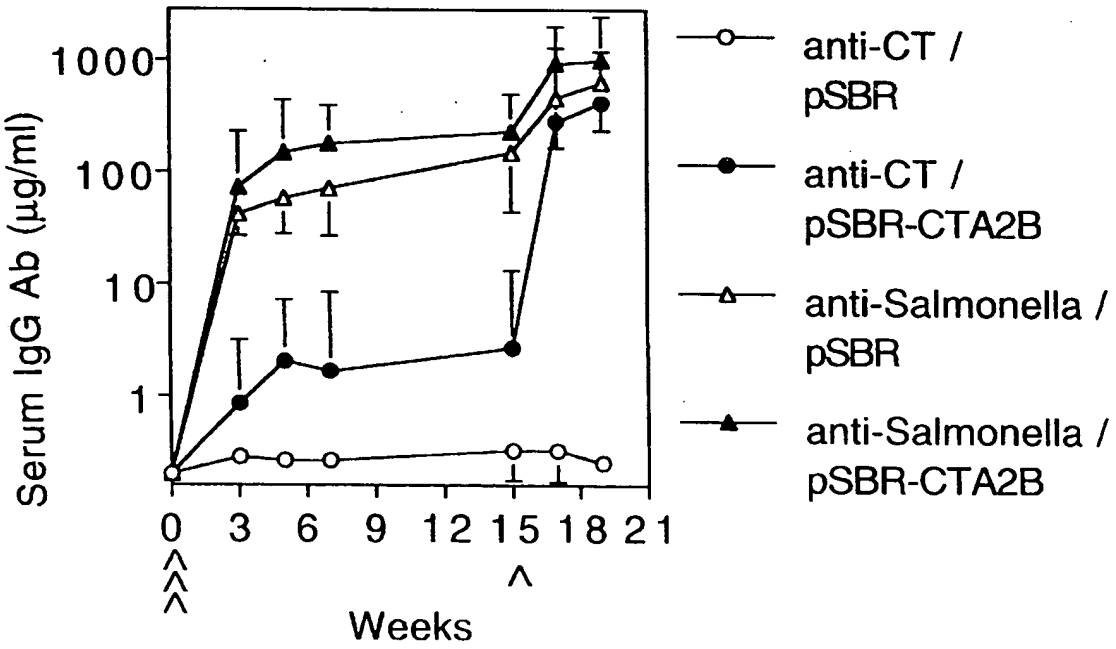

Fig. 11B

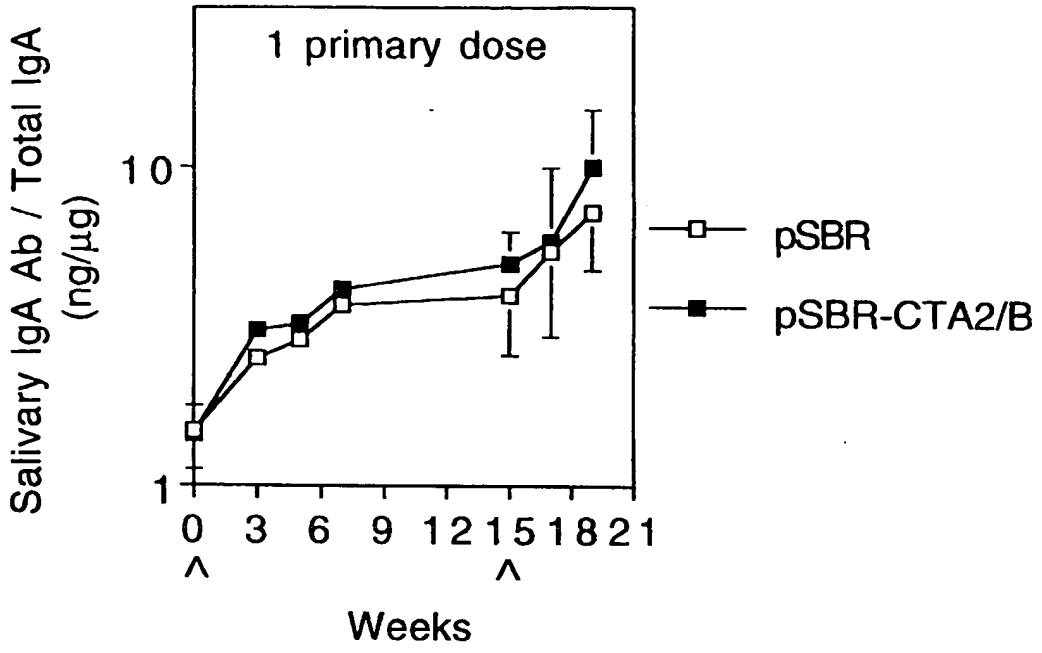

Fig. 12A

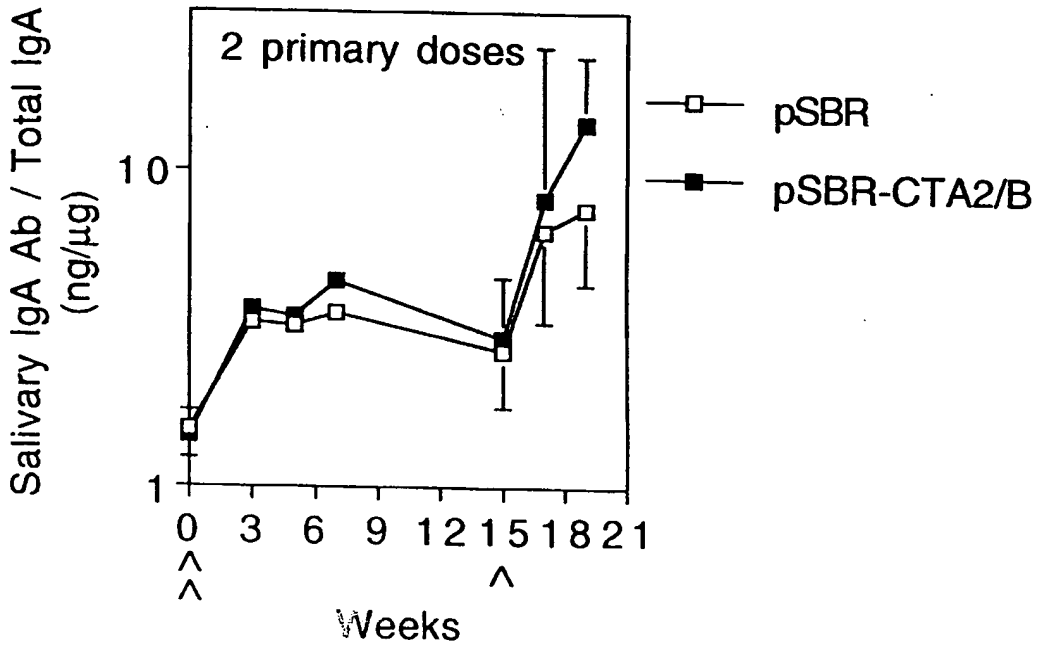

Fig. 12B

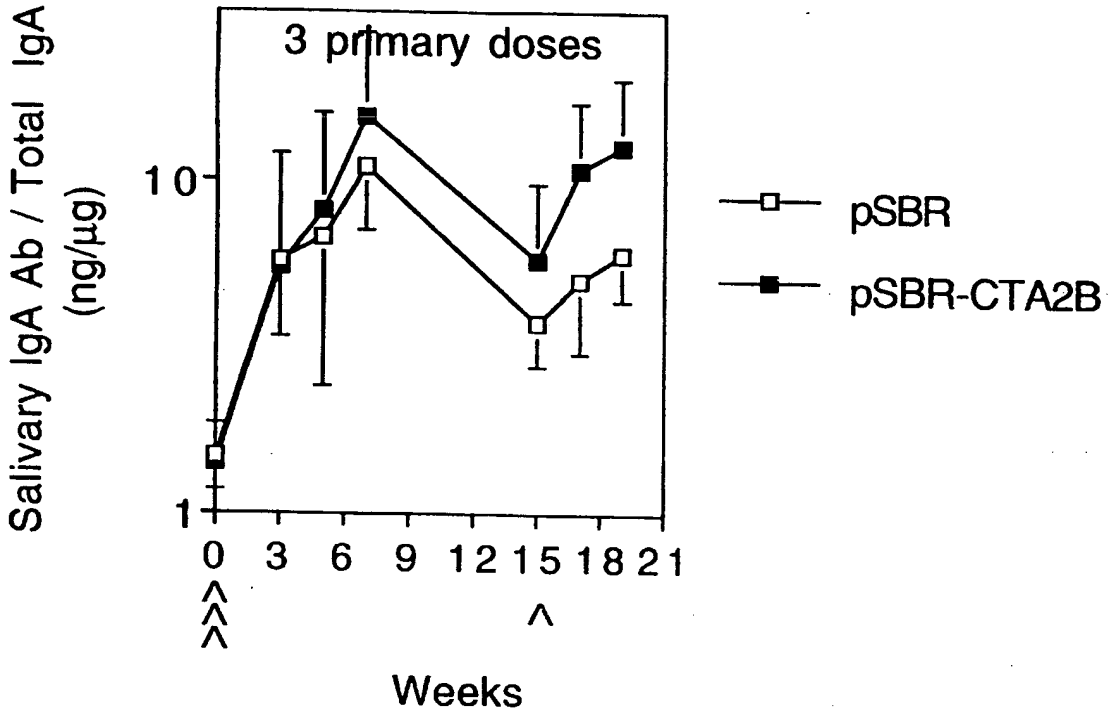
3 primary doses
Salivary IgA Ab / Total IgA (ng/µg)
10
1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2B

Fig. 12C

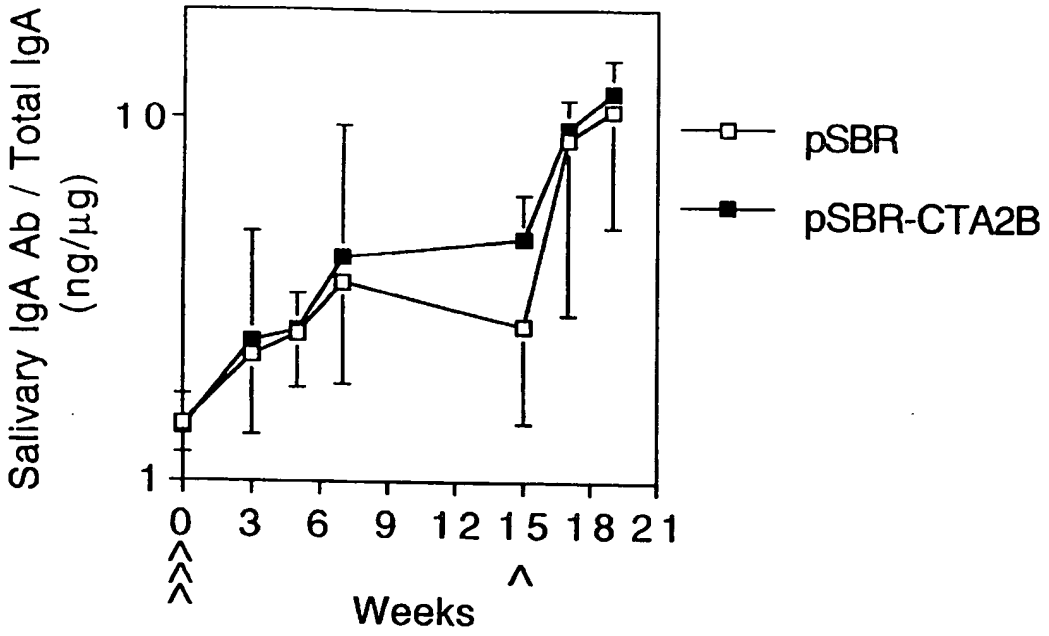

Fig. 13A

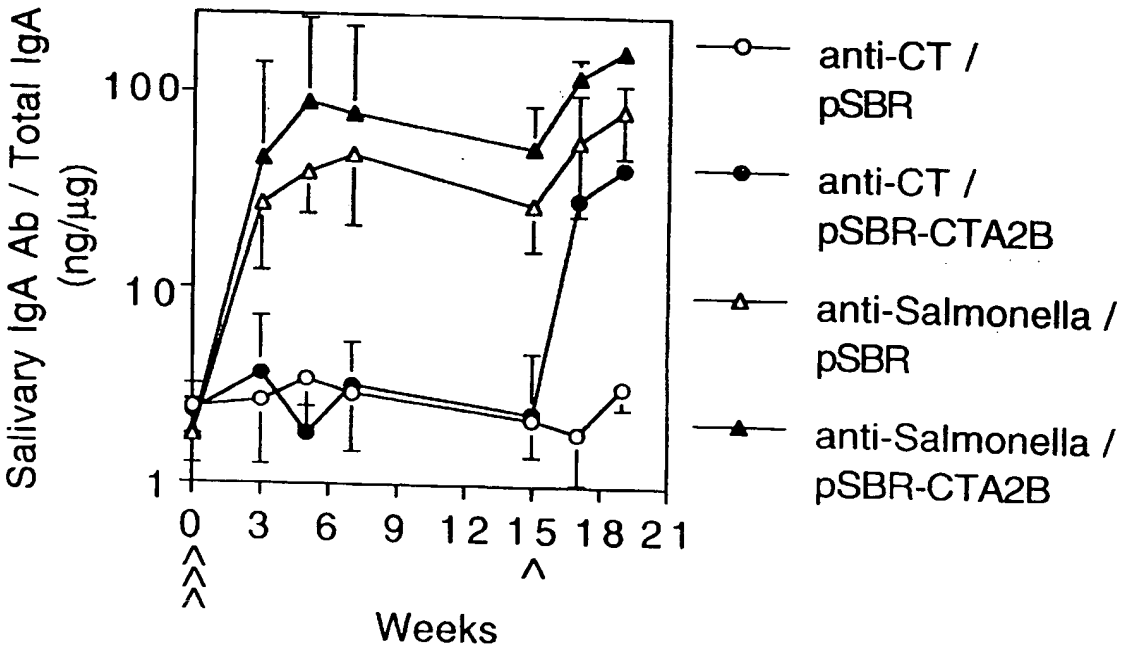

Fig. 13B

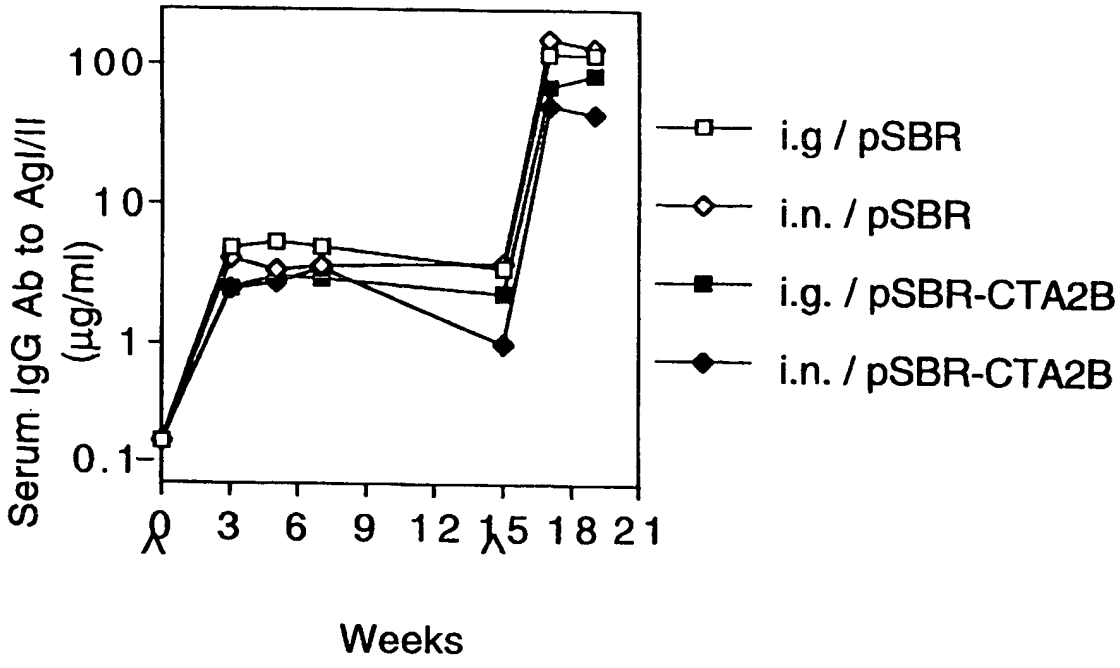

Fig. 14A

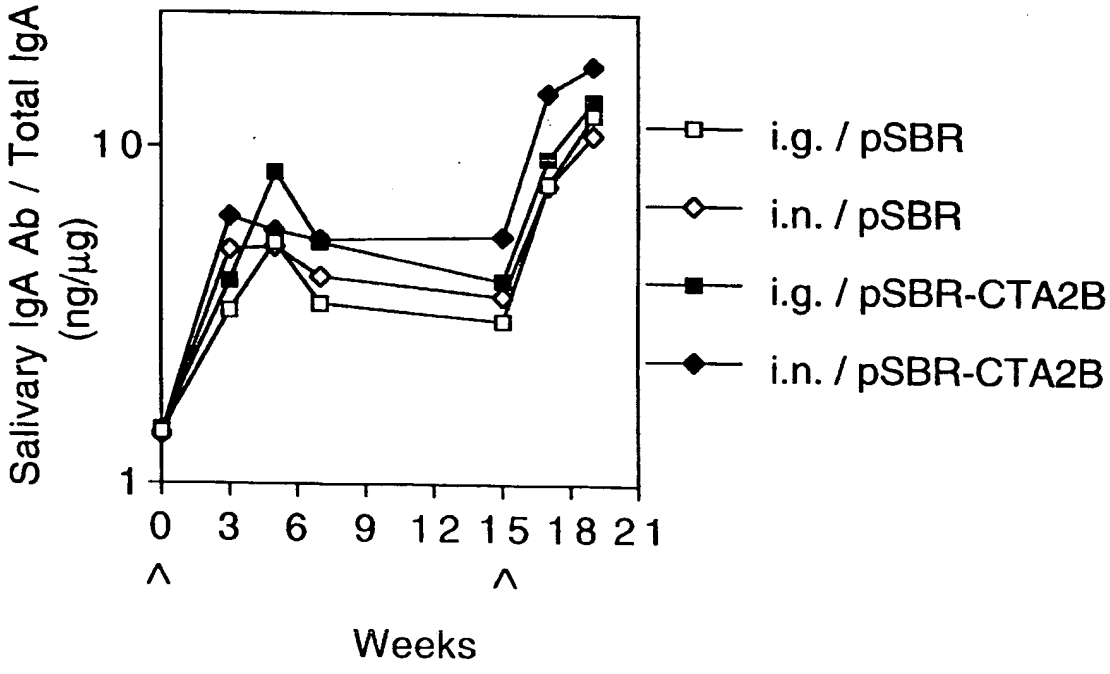

Fig. 14B

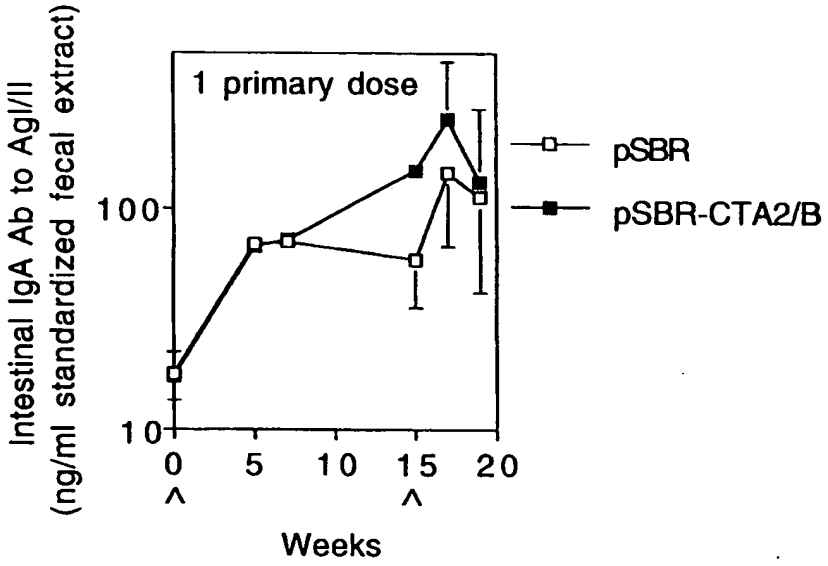

Fig. 15A

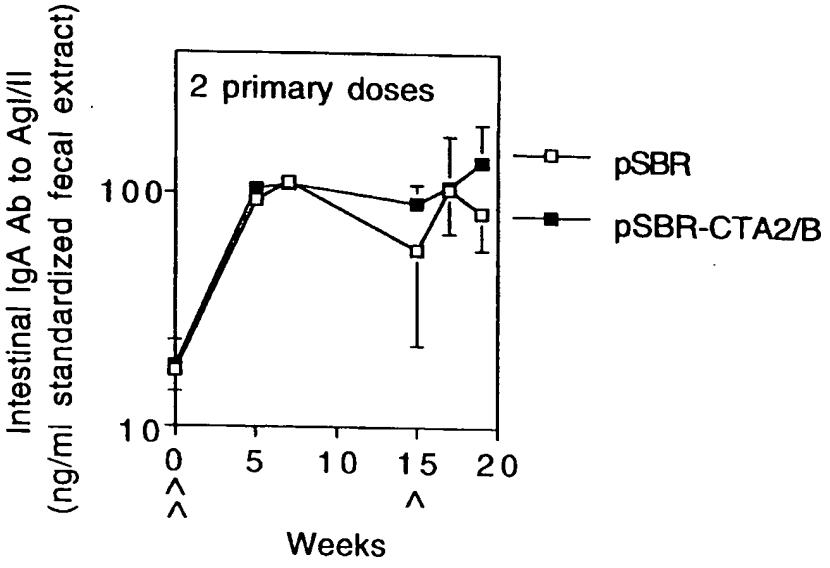

Fig. 15B

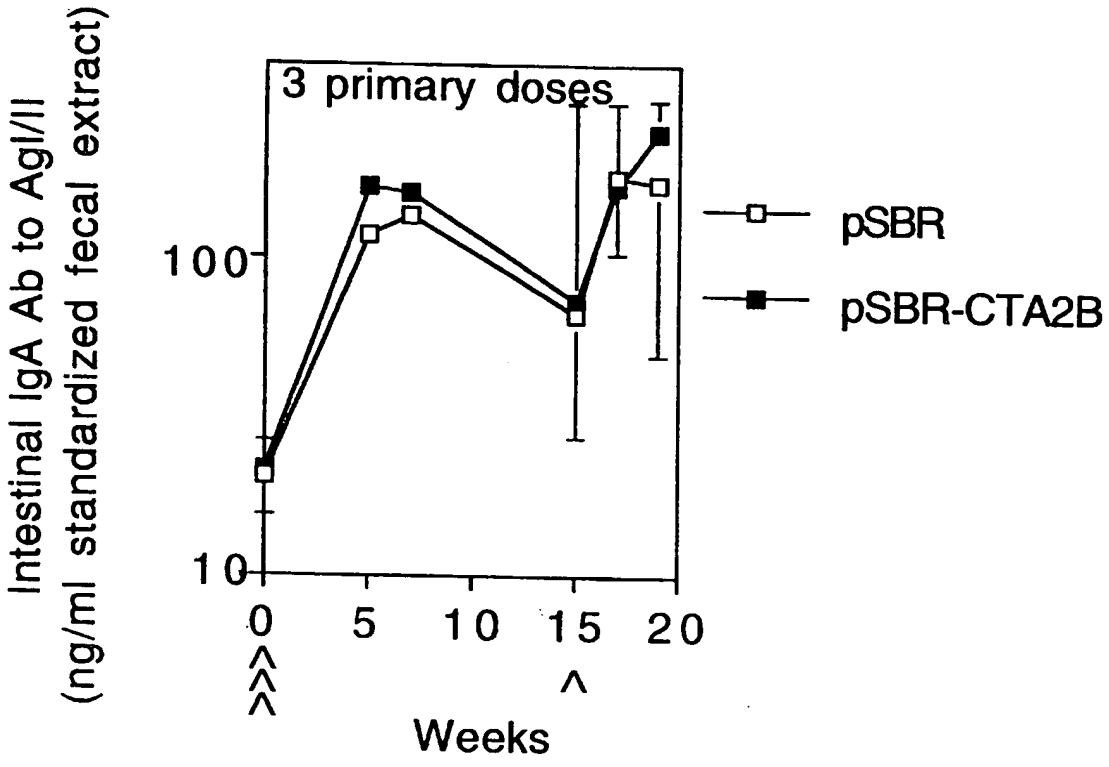
Intestinal IgA Ab to AgI/II
(ng/ml standardized fecal extract)
3 primary doses
100
10
0
5
10
15
20
Weeks
pSBR
pSBR-CTA2B

Fig. 15C

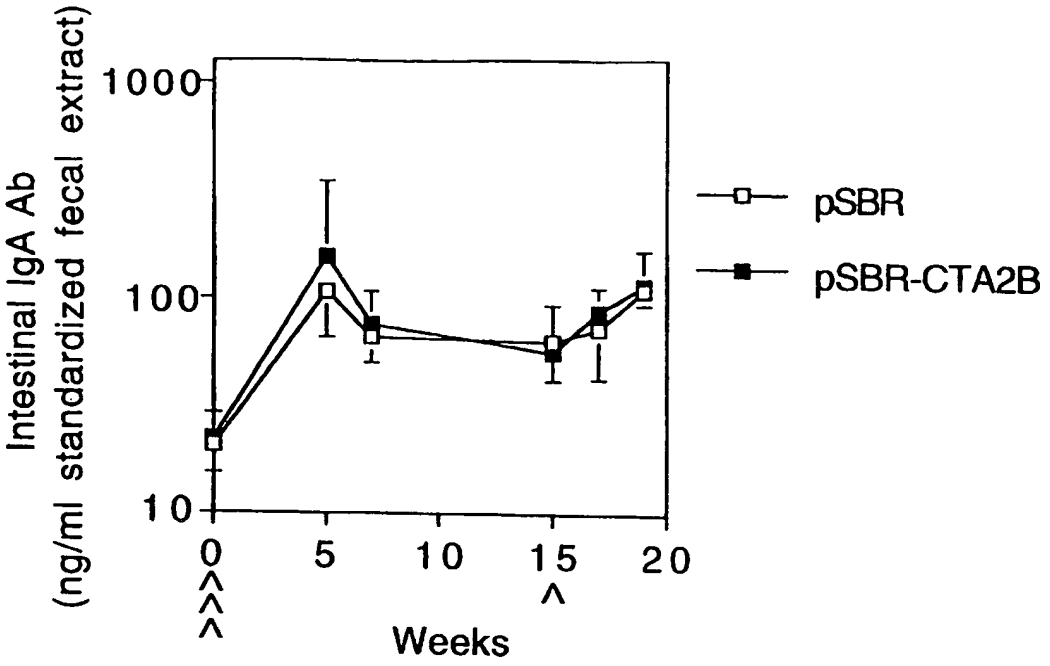

Fig. 16A

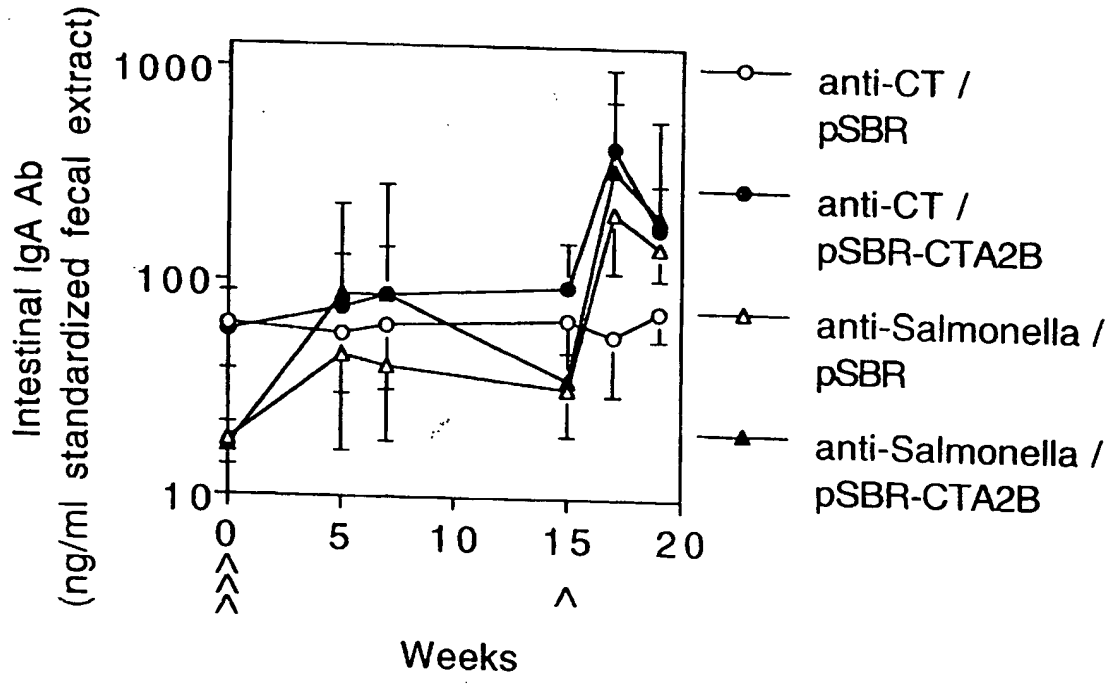

Fig. 16B

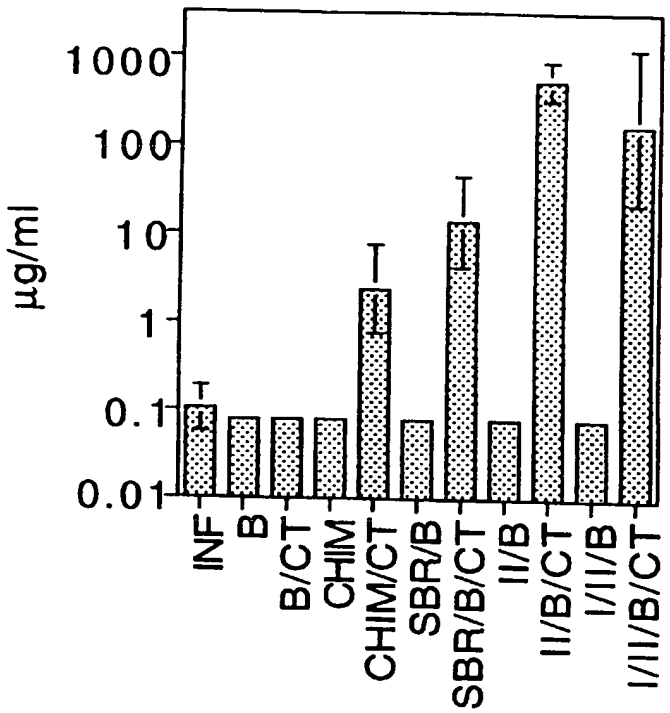
1000
100
10
1
0.1
0.01
µg/ml
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 17

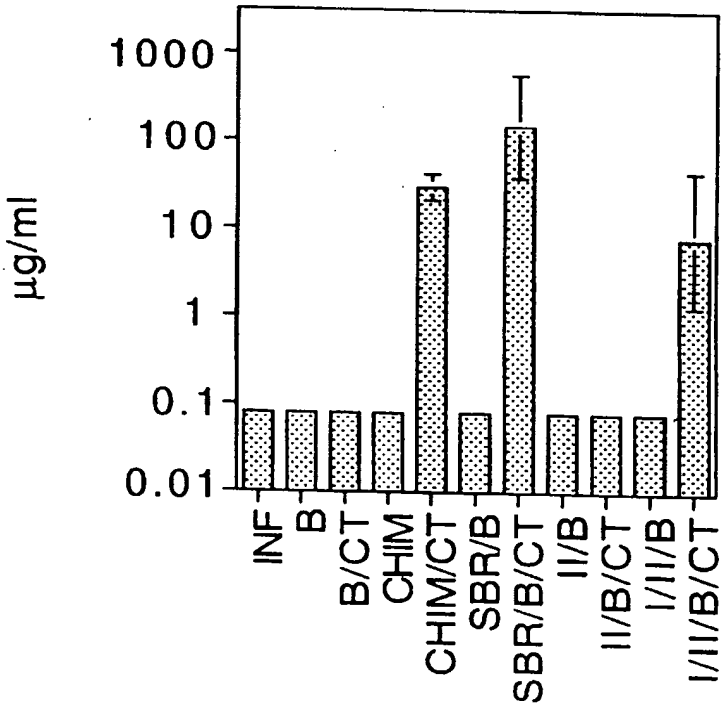
1000
100
10
1
0.1
0.01
µg/ml
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 18

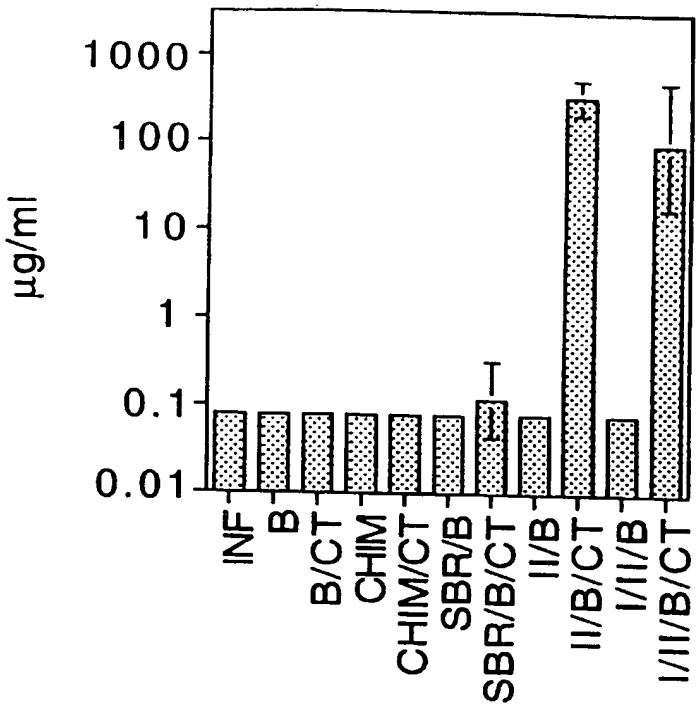
µg/ml
1000
100
10
1
0.1
0.01
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 19

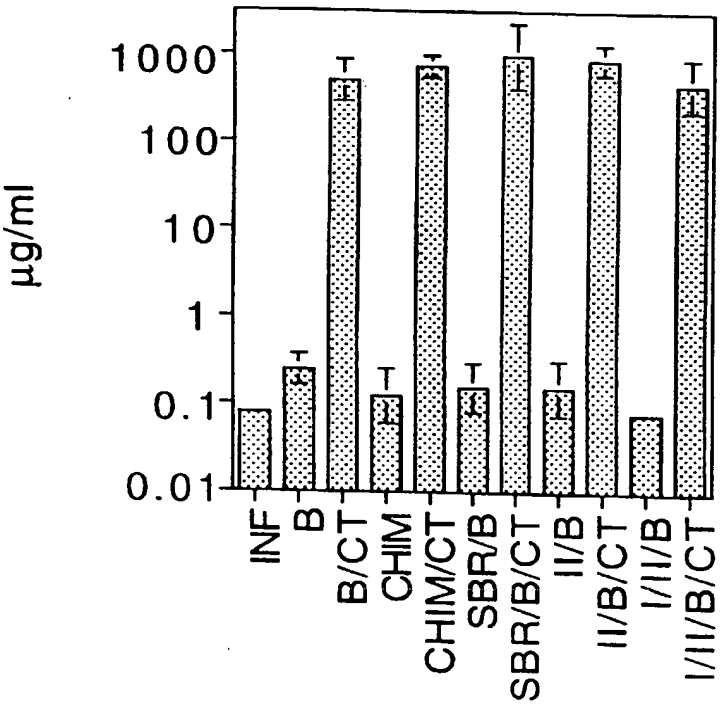
µg/ml
1000
100
10
1
0.1
0.01
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 20

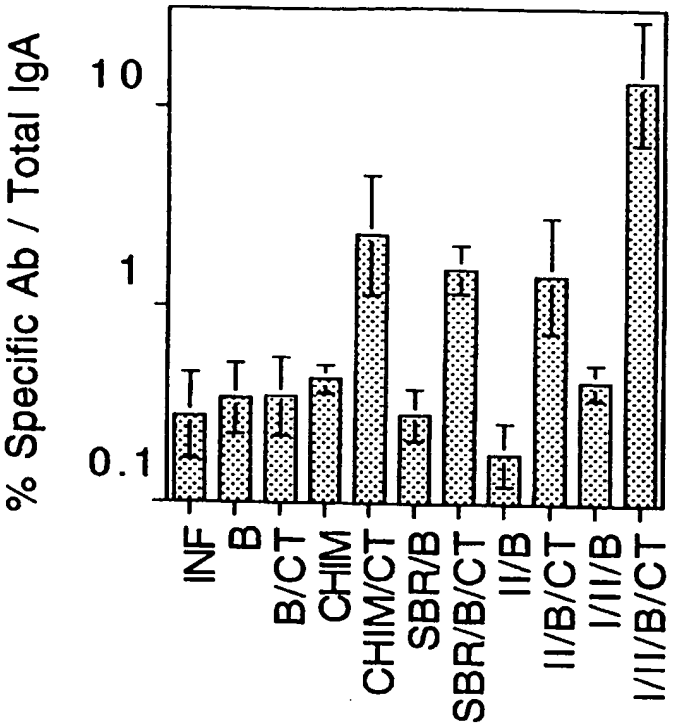
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 21

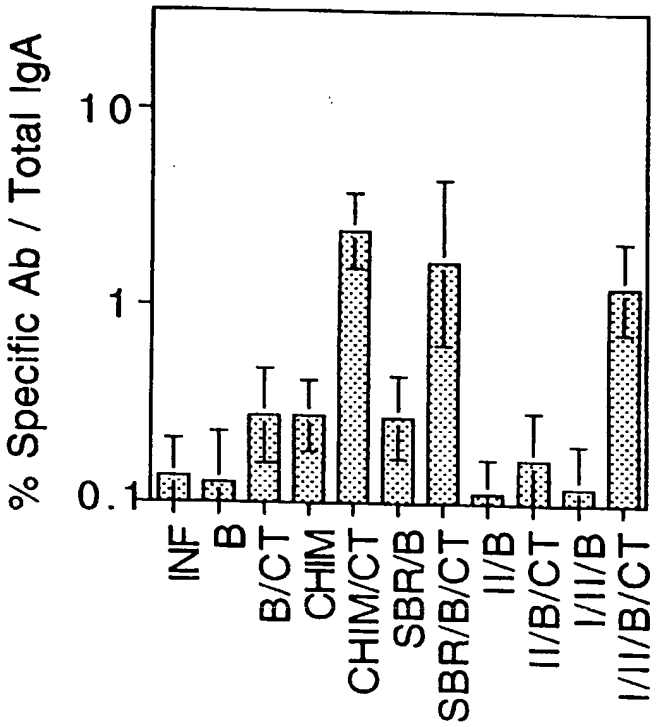
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 22

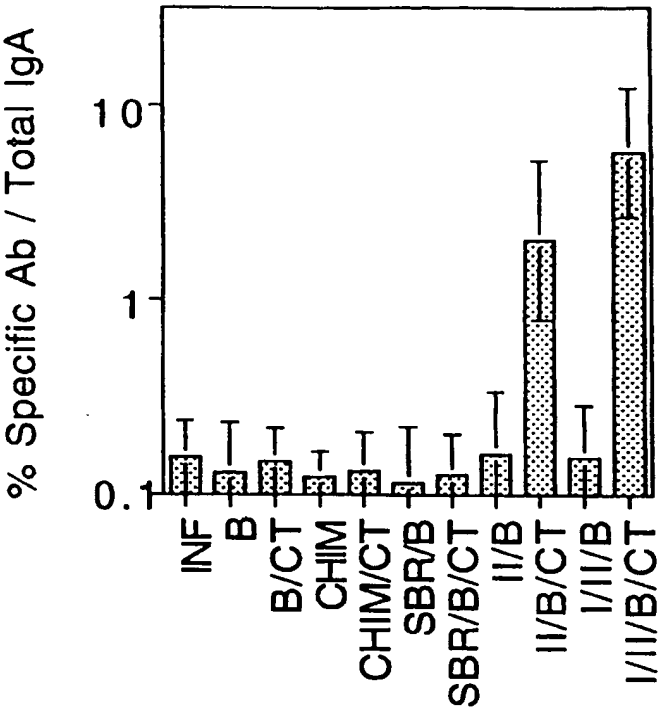
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 23

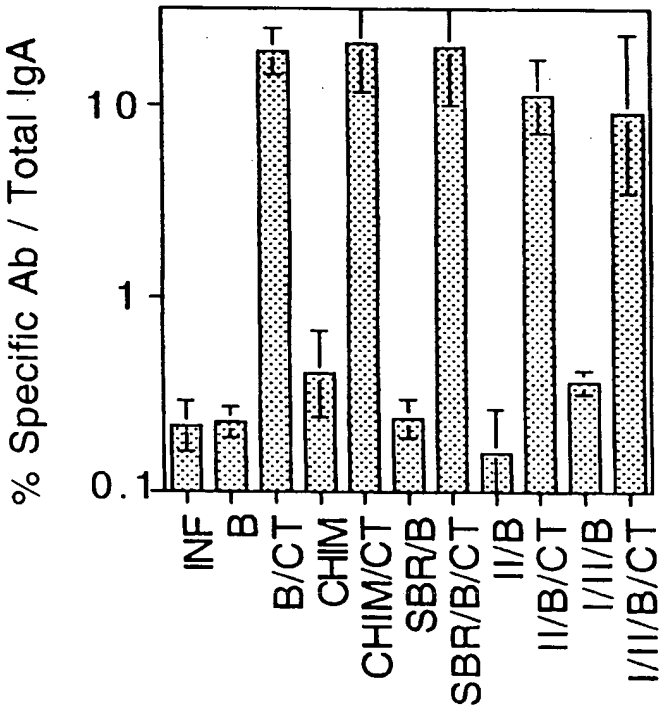
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 24

MUCOSAL IMMUNOGENS FOR NOVEL VACCINES

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to provisional application U.S. Ser. No. 60/024,074, filed Aug. 16, 1996, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of molecular immunology and protein chemistry. More specifically, the present invention relates to a novel mucosal immunogens for use in novel vaccines.

2. Description of the Related Art

An oral immunization strategy is when the desired mucosal immunogen is genetically fused to the A2 subunit of cholera toxin (CT) that mediates association with the B subunit of CT, a potent immunoenhancing agent. An antigen selected for evaluating the oral immunogenicity of such non-toxic CTA2/B-based constructs is the saliva-binding region (SBR) of the AgI/II adhesin from the oral bacterium *Streptococcus mutans*. The SBR genetically linked to CTA2/B, designated SBR-CT^{M1}, was found to be immunogenic by the oral route and elicited high levels of secretory immunoglobulin A (S-IgA) and serum IgG antibodies to AgI/II.

Despite its great importance for mucosal defense, the S-IgA antibody response is often of relatively short duration, lasting from a few weeks in experimental animals to a few months in humans. Moreover, whether the secretory immune system is capable of anamnestic immune responses has been debated, but recent studies in mice and humans have addressed the concept of immunological memory at the mucosal surfaces. Immunological memory can be manifested as a long-lasting immune response or as a faster and more vigorous anamnestic response to re-encounter with an antigen. A desirable vaccine characteristic is the induction of prolonged immune responses, especially when the pathogenic organism is frequently encountered at mucosal surfaces, in which case a continuing level of immunity may be necessary.

IgA antibodies in external secretions protect mucosal surfaces, e.g., of the gastrointestinal and respiratory tracts, by blocking microbial adherence and colonization. Oral administration of vaccines can result in the induction of secretory immune responses after uptake of the antigen by the gut-associated lymphoid tissues, a major IgA inductive site. However, most soluble proteins are not only poor immunogens when given orally but they may induce a state of systemic unresponsiveness known as "oral tolerance". The experimental use of cholera toxin from *Vibrio cholerae* or the related heat-labile enterotoxin from *Escherichia coli* as mucosal adjuvants inhibit induction of oral tolerance and potentiates the immune responses to co-administered protein antigens.

Another strategy to overcome problems associated with oral immunization (e.g., denaturation of the protein immunogens by gastric acid and digestive enzymes, limited absorption by the intestinal mucosa, and clearance by peristalsis) as well as the need to purify a vaccine protein, involves the use of avirulent derivatives of *Salmonella typhimurium* as a vaccine delivery system with tropism for the gut-associated lymphoid tissues. Oral immunization with avirulent *S. typhimurium* expressing heterologous antigens is generally not associated with suppression but rather with stimulation of protective secretory and serum antibody responses as well as cell-mediated immune responses.

Initial adherence of *Streptococcus mutans* to tooth surfaces appears to be mediated largely by the 167 kDa surface fibrillar adhesin known as AgI/II (synonyms: antigen B, P1, SpaP, PAc). The adhesion domain that interacts with salivary pellicle has been located to the alanine-rich (A) repeat region in the N-terminal part of the molecule extending from the cell surface probably in an α-helical conformation. Studies on AgI/II indicated that rhesus monkeys immunized with *S. mutans* and protection against dental caries mounted antibody responses especially against the complete molecule rather than against AgII, which corresponds to the C-terminal one-third. These results were supported by the finding that immunization with either complete AgI/II, or the isolated AgI component (corresponding to the N-terminal two-thirds), afforded protection against caries. Thus, one approach to immunization against *S. mutans*-induced dental caries can be based upon the generation of an appropriate antibody response in the saliva that would inhibit the adherence of *S. mutans* to tooth surfaces. Human secretory IgA (S-IgA) antibodies to AgI/II inhibit such adherence. However, S-IgA antibodies in saliva and other secretions are not effectively induced by conventional parenteral immunization.

S-IgA antibodies are most effectively induced by stimulating the common mucosal immune system, for example, by enteric immunization which stimulates the gut-associated lymphoid tissues including the Peyer's patches (PP) of the small intestine. Considerable attention has been given to the development of improved procedures for the oral delivery of vaccines, one of which is coupling antigens to the nontoxic binding B subunit of cholera toxin (CT), a safe and highly immunogenic protein in humans. CTB, because of its avid binding to G_{M1} ganglioside, present on all nucleated cell surfaces, is readily taken up by the M cells covering PP, and passed to the underlying immunocompetent cells which initiate the mucosal IgA antibody response. Antigen-stimulated IgA-committed B cells, and corresponding T helper cells, then emigrate via draining lymphatics to the mesenteric lymph nodes (MLN) and thence via the thoracic duct to the circulation before relocating in the effector sites of mucosal immunity, such as the salivary glands. Terminal differentiation of B cells into IgA-secreting plasma cells occurs here and their product, polymeric IgA is transported through the glandular epithelium to form S-IgA. Other antigens can be coupled to CTB to generate strong mucosal IgA antibody responses to the desired antigen and that intact CT, though toxic, serves as an adjuvant that enhances the response to co-administered antigens.

The expression of foreign genes encoding immunogens of interest in avirulent derivatives of *Salmonella typhimurium* is used as a strategy to induce mucosal immune responses to protein Ags which are usually poor oral immunogens when administered alone. Indeed, *S. typhimurium* appears to be an effective antigen delivery system because of its ability to colonize the gut-associated lymphoid tissue where secretory IgA responses are initiated (1). Electron microscopy studies have shown that *S. typhimurium* preferentially interacts with the specialized antigen-sampling M cells overlying the Peyer's patches in the GALT (2). At these sites, antigenic stimulation of specific IgA-committed B cells results in their migration to mucosal tissues where they differentiate into IgA-secreting plasma cells, with subsequent release of secretory IgA antibodies in external secretions (3). These antibodies play an important role in the defense of mucosal

surfaces, e.g., of the gastrointestinal and respiratory tracts, by inhibiting microbial adherence and colonization or invasion (4). Depending on the species and host, Salmonella organisms may disseminate to the spleen, the liver, and regional lymph nodes, take residence in macrophages, and thereby induce serum antibody and cellular immune responses (1).

The issue of whether CTB alone has mucosal adjuvant properties has been questioned especially for oral immunization (14, 19), although CTB confers a targeting property to Ags coupled to it because of its affinity for G_{M1} ganglioside receptors (20). If CTB possesses immunoenhancing properties, other than its carrier/targeting effect, it could also be useful as a Salmonella-expressed adjuvant, especially for proteins that are poor immunogens even when delivered by *S. typhimurium*. A commercially obtained CTB preparation, lacking detectable cAMP-elevating capacity, was found to potentiate in vitro antibody production against an unrelated protein antigen by stimulating the antigen-presenting function of splenic adherent cells through enhanced IL-1 production (21). An enhancing effect on antigen presentation by macrophages was also demonstrated for recombinant (r)CTB (22), which, moreover, up-regulates expression of MHC class II molecules on B cells, which can also act as antigen-presenting cells (23). The fact that commercially available CTB is contaminated with small but variable amounts of intact CT may explain conflicting reports on the adjuvant capacity of CTB (14) as well as findings that commercial CTB is superior to rCTB as an adjuvant for intranasal (i.n.) immunization (24, 25).

The prior art is deficient in the lack of effective mucosal immunogens, for use in, e.g., a caries vaccine. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention demonstrates that primary oral immunization of mice with a bacterial protein antigen genetically coupled to the A2/B subunits of cholera toxin induced specific secretory immunoglobulin A and serum IgG antibodies that persisted at substantial levels for at least 11 months. A subsequent single booster immunization did not further enhance the antibody responses. Long-term antibody persistence may be especially important in infections caused by common pathogens for which continuous immunity would be advantageous.

The present invention further shows that a major adhesin from the oral pathogen *Streptococcus mutans* is mucosally immunogenic upon genetic fusion with the cholera toxin A2/B subunits. To take advantage of the ability of *Salmonella typhimurium* to deliver cloned antigens to the mucosal inductive sites that would obviate the need for antigen purification, this chimeric construct was expressed in an attenuated *S. typhimurium* strain under the control of bacteriophage T7 transcription. Residual expression of the temperature-regulated T7 RNA polymerase at 30° C. allowed production of the chimeric protein at 2–3% of the total soluble protein, but it was increased 5–6 times following induction at 37° C. Oral administration of a single dose of 10^9 recombinant Salmonella to mice resulted in serum IgG and salivary IgA antibody responses to Salmonella, cholera toxin, and the streptococcal adhesin, which were generally enhanced after a booster immunization.

The present invention also discloses an avirulent *Salmonella typhimurium* vaccine strain expressing a streptococcal protein adhesin, and a similar clone which produces the same streptococcal antigen linked to the cholera toxin A2/B subunits, which were compared for their ability to induce antibody responses to the expressed heterologous antigen after oral or intranasal immunization of mice. Expression of cloned immunogens in these systems is temperature-regulated, being optimal at 37° C., and the two clones produced similar levels of the streptococcal antigen. Both clones were found to stimulate high levels of serum IgG and mucosal IgA antibodies to the cloned immunogen. A consistent trend was observed towards higher mucosal IgA but lower serum IgG responses in the case of the *S. typhimurium* vector that co-expressed CTA2/B, a potential mucosal adjuvant, regardless of the route of administration. Also noteworthy was the capacity of these antigen-delivery systems to induce anamnestic mucosal and systemic responses to the cloned immunogen 15 weeks after the primary immunization, despite pre-existing immunity to the Salmonella vectors. Although the serum IgG response against the Salmonella vector was characterized by a high IgG2a/IgG1 ratio (indicative of the Th1/Th2 profile), a mixed IgG1 and IgG2a pattern was observed for the carried heterologous antigen, which displayed a dominant IgG1 response when administered as a purified immunogen. The present invention indicates that the recombinant streptococcal antigen and CTA2/B are strong immunogens when expressed by the antigen-delivery system, and that CTA2/B may have an additional immunoenhancing activity in the mucosal compartment besides its ability to target antigen uptake into the mucosal inductive sites and, therefore, may be useful as a *S. typhimurium*-cloned adjuvant for co-expressed protein Ags.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

FIGS. 1(A)–1(D) shows the persistence of serum IgG antibody to AgI/II and CT after peroral immunization of mice with SBR-CT^{AA1} chimeric protein (^^) and a single booster immunization 11 months later (^). Mice were given the immunogen in the presence (FIG. 1A and FIG. 1C) or absence FIG. 1B and FIG. 1D of CT adjuvant. Data are presented for each mouse individually.

FIGS. 2(A)–2(D) shows the duration of salivary IgA antibody to AgI/II and CT following peroral immunization of mice with SBR-CT^{AA1} chimeric protein (^^) and a single booster immunization 11 months later (^). Mice were given the immunogen in the presence FIG. 2A and FIG. 2C or absence FIG. 2B and FIG. 2D of CT adjuvant. Data are presented for each mouse individually.

FIG. 3 shows the SDS-PAGE analysis of inclusion bodies produced under different induction conditions by *S. typhimurium* expressing the SBR-CT^{AA1} chimeric protein. A related strain lacking SBR-CT^{AA1} was used as a negative control. Two prominent bands migrating at about 60 kDa and about 14 kDa present in the expressing strain after 42° C. induction (and to a lesser extent after 37° C. induction)

correspond to the Mr of SBR-CTA2 and CTB (monomer), respectively. The CTB component of purified SBR-CT$^{\Delta A1}$ ran relatively faster (Mr~11.5 kDa) due to processing of the precursor polypeptide by signal peptidase during transport to the periplasmic space, while an additional band from SBR-CTA2 may represent a degradation product.

FIGS. 4(A)–4(B) shows the serum IgG FIG. 4A and salivary IgA FIG. 4B antibody responses to *S. typhimurium* and cloned antigens in mice orally immunized with 10^9 bacteria on weeks 0 and 7 (^). Immune response data for weeks 5, 8 and 10 represent geometric means ×/+ standard deviation of five mice. Pooled samples were assayed at other time points.

FIGS. 5(A)–5(B) show the serum IgG FIG. 5A and salivary IgA FIG. 5B antibody responses to AgI/II in unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Immunizations were given on days 0, 10, and 20, and samples were collected 10 days after each immunization, i.e., on days 0 (unimmunized mice), 10 (one dose), 20 (2 doses), or 30 (3 doses). Results shown are mean+/–SD of samples from 3 animals analysed separately. Salivary IgA antibodies were below detectable levels (<0.1 μg/ml) on days 0 and 10, and are shown at this level in (b).

FIG. 6 shows the proliferative responses of cells from PP, MLN, and spleens of unimmunized (control) mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant, cultured in vitro with AgI/II. Results shown are mean stimulation indices of 3 replicate cultures; SD values ranged from –0.04 to –0.95.

FIG. 7 shows the phenotypic analysis of cells from PP, MLN, peripheral blood, and spleen of unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Each 'pie' shows the proportions of $CD4^+$, $CD8^+$, and $CD3^-$ (non-T) cells as a percentage of total gated mononuclear cells determined by flow cytometry, starting with unimmunized mice (center of each 'pie'), and proceeding outwards in concentric rings with mice immunized once, twice, or three times. Numbers within the rings are the % of each phenotype of cells (for clarity, $CD8^+$ cell data are shown outside the 'pies' in descending order: 0, 1, 2, and 3 doses); the value shown for MLN from mice immunized once with SBR (marked as "51?") was not obtained experimentally, but was inserted for plotting purposes as the average of the values either side of it. The numbers in each ring of a 'pie' do not sum to exactly 100%, because of the presence of some $CD4^-/CD8^-$ ("double-negative") $CD3^+$ T cells, and possibly some $CD4^+/CD8^+$ ("double-positive") T cells in each cell preparation.

FIG. 8A shows a schematic representation of the plasmids used to transform SBR-CT$^{\Delta A1}$-or SBR-expressing *S. typhimurium* clones. FIG. 8B shows a western blotting of cell lysates from the SBR-CT$^{\Delta A1}$-and the SBR-producing clones using antibodies to SBR detected the SBR-CTA2 fusion protein and the SBR, respectively. The control lane is a lysate from a clone transformed with the pGP1-2 plasmid only.

FIGS. 9(A)–9(C) shows the serum IgG antibody responses to native AgI/II in mice orally immunized (^) on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* clones. During week 0 mice were immunized with one FIG. 9A, two FIG. 9B, or three FIG. 9C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Data represent geometric means ×/(SD of 5 to 6 mice. For clarity only the upper or lower SD bars are shown.

FIGS. 10(A)–10(C) shows the serum IgG (FIG. 10A), salivary IgA (FIG. 10B), and intestinal IgA (FIG. 10C) antibody responses to CT and *S. typhimurium* vector after oral immunization (^) of mice with SBR- or SBR-CT$^{\Delta A1}$-producing Salmonella clones on weeks 0 and 15. Mice were immunized with two doses of 10^9 CFU of the appropriate *S. typhimurium* clone during week 0, and were given a single dose of 10^{10} CFU 15 weeks later. Results are shown as geometric means ×/+ SD of 5 to 6 mice. Data for weeks 3 to 7 (FIGS. 10B and 10C) were obtained by assaying pooled samples from 5 to 6 mice per corresponding group. For clarity only the upper or lower SD bars are shown.

FIGS. 11(A)–11(B) shows the serum IgG antibody responses to AgI/II (FIG. 11A) and CT and Salmonella vector (FIG. 11B) in mice immunized (^) by the i.n. route on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S.typhimurium* clones. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data are expressed as the geometric means ×/(SD of 6 mice.

FIGS. 12(A)–12(C) shows the salivary IgA antibody responses to AgI/II in mice orally immunized (^) on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S.typhimurium*. During week 0 mice were immunized with one FIG. 12A, two FIG. 12B, or three FIG. 12C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Results are the geometric means ×/(SD of 5 to 6 mice. Data for weeks 3, 5, and 7 in groups which received one or two primary doses, were obtained by assaying pooled samples from 5 to 6 mice per corresponding group.

FIGS. 13(A)–13(B) shows the salivary IgA antibody responses to AgI/II (FIG. 13A) and CT and Salmonella vector (FIG. 13B) after i.n. immunization (^) of mice on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* clones. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data represent geometric means ×/(SD of 6 mice.

FIGS. 14(A)–14(B) shows the serum IgG FIG. 14A and salivary IgA FIG. 14B antibody levels to AgI/II induced by either i.n. or i.g. immunization (^) of mice on weeks 0 and 15 with *S. typhimurium* expressing SBR alone or SBR-CT$^{\Delta A1}$ chimeric protein. The animals were given a single primary immunization of 10^9 CFU (i.n.) or 10^{10} CFU (i.g.) followed by a booster immunization with the same dose 15 weeks later. Data were obtained by assaying pooled samples from 3 mice per corresponding group.

FIGS. 15(A)–15(C) shows the intestinal IgA antibody responses to AgI/II in mice orally immunized (^) on weeks 0 and 15 with *S. typhimurium* clones expressing SBR or SBR-CT^{DA1}. During week 0 mice were immunized with one FIG. 15A, two FIG. 15B, or three FIG. 15C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Results are the geometric means ×/(SD of 5 to 6 mice. Data for weeks 5 and 7 were obtained by assaying pooled samples from 5 to 6 mice per corresponding group.

FIGS. 16(A)–16(B) shows the intestinal IgA antibody responses to AgI/II (FIG. 16A) and CT and *S. typhimurium* (FIG. 16B) after i.n. immunization (^) of mice on weeks 0 and 15 with *S.typhimurium* vectors producing SBR alone or

SBR-CT$^{\Delta A1}$ chimeric molecule. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data represent geometric means ×/(SD of 6 mice.

FIG. **17** shows the serum IgG antibody responses to native AgI/II in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of recombinant B subunit of cholera toxin (rCTB) or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of cholera toxin (CT). Data are from samples obtained two weeks after the last immunization and represent geometric means±standard deviation (SD).

FIG. **18** shows the serum IgG antibody responses to recombinant SBR (42-kDa saliva-binding region at the N-terminal of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definitions) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **19** shows the serum IgG antibody responses to AgII (C-terminal one-third of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent means±SD.

FIG. **20** shows the serum IgG antibody responses to CT in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **21** shows the salivary IgA antibody responses to native AgI/II in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of recombinant B subunit of cholera toxin (rCTB) or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of cholera toxin (CT). Data are samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **22** shows the salivary IgA antibody responses to recombinant SBR (42-kDa saliva-binding region at the N-terminal of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are samples obtained two weeks after the last immmunization and represent geometric means±SD.

FIG. **23** shows the salivary IgA antibody responses to AgII (C-terminal one-third of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **24** shows the salivary IgA antibody responses to CT in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

Group Abbreviations	Groups Defined
INF	Unimmunized controls
B	rCTB
B/CT	rCTB + CT
CHIM	SBR-CT$^{\Delta A1}$
CHIM/CT	SBWCT$^{\Delta A1}$ + CT
SBR/B	SBR-rCTB
SBR/B/CT	SBR-rCTB + CT
II/B	AgII-rCTB
II/B/CT	AgII-rCTB + CT
I/II/B	AgI/II-rCTB
I/II/B/CT	AgI/II-rCTB + CT

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations may be used herein: GALT: gut-associated lymphoid tissue; CT: cholera toxin; CTA2/B: cholera toxin subunits A2/B; CTB: cholera toxin subunit B; rCTB:recombinant CTB; i.n.: intranasal; i.g.: intragastric.

As used herein, the term "fusion protein" refers to a single contiguous protein produced by the expression of DNA sequences for one protein fused to DNA sequences encoding a different protein.

As used herein, the term "chimeric protein" refers to a fusion protein assembled with a different protein.

The present invention is directed to a composition of matter comprising a novel plasmid for use as a mucosal immunogen to prevent or inhibit the formation of dental caries. This recombinant plasmid expresses a chimeric protein which is a primary immunogen that induces long term antibody responses. Production of the chimeric protein is optimal at physiological temperatures, i.e., 37° C.

In one embodiment of the present invention, the chimeric protein immunogen is constructed by fusing a large segment of a protein antigen, e.g., Salivary Binding Protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) to the A2 subunit of cholera toxin, and assembling this with cholera toxin B subunits to form the chimeric protein. This is designated SBR-CTA2/B, or SBR-CT$^{\Delta A1}$. The latter designation refers to the deletion the A1 subunit of cholera toxin (which is the actual toxic component) from the genetic construct, and its replacement by SBR. "SBR-CTA2/B" is a shorthand molecular formula for the chimeric protein.

Initially, SBR-CTA2/B was produced in *Escherichia coli*, and the purified chimeric protein, was immunogenic by oral or intranasal administration with the generation of serum and salivary antibodies which can last for up to at least 11 months in mice. The duration of antibody responses is novel, and not predictable or expected. The establishment of regulatory T cells is in part intended to build a case for the generation of long-term memory within the mucosal immune system, because that is not 'expected' insofar as is

widely held that memory is limited in the mucosal immune system. The chimeric protein of the present invention, when expressed in attenuated *Salmonella typhimurium* produces significant increases in serum IgG and salivary IgA antibody levels after oral immunization.

A second embodiment of the present invention is the expression of SBR-CTA2/B in *Salmonella typhimurium* for delivery in a live carrier (attenuated) organism. The advantages of this construct are that there is no need to purify the product, and that a slightly different spectrum of immune responses may be obtained, with beneficial applications in some diseases.

In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin A2/B chimeric protein expressed in *E. coli*. Intragastric immunization of salivary binding protein coupled to CTB in this chimeric protein form leads to increased antigen responsive T cells.

In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin$^{\Delta A1}$ chimeric protein expressed in *Salmonella typhimurium*. Oral immunization using this recombinant plasmid results in increased serum IgG responses to antigen. Oral immunization using this recombinant plasmid also resulted in increased salivary IgA antibody responses to antigen.

There are many potential uses for the technology of the present invention in mucosal vaccine development. The basic method is amenable to almost any other protein antigen that can be cloned and inserted into the construct instead of SBR. For example, a protein antigen from *Streptococcus pneumoniae* can be used to make a potential vaccine against pneumonia. Similarly, constructs from group A streptococcal proteins or a vaccine against *Helicobacter pylori* can be prepared using the methodology disclosed in the instant specification. Various applications of the present invention can be incorporated into commercial products, i.e., vaccines for the generation of immune responses that would afford protection against infections, or various modifications of the immune response. These are based on the use of CTA2/B chimeric proteins that include protein segments from a variety of microorganisms, intended for administration orally or intranasally, or possibly by other mucosal routes (e.g., rectally or intra-vaginally).

For example, one may prepare vaccines to generate immunity to the organisms responsible for dental caries, i.e., the "*mutans*" streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*). This is based on the saliva-binding region of *S. mutans* AgI/II, as described above. Secondly, one may prepare vaccines against *Streptococcus pyogenes* ("strep. throat and its sequelae including acute rheumatic fever and acute glomerulonephritis, scarlatina, streptococcal toxic shock, and other infections). Further, one may prepare: vaccines against *Streptococcus pneumoniae* (pneumococcal pneumonia, otitis media, meningitis) using sequences from pneumococcal surface proten A (PspA), vaccines against: a) *neisseria meningitidis* (meningococcal meningitis, otitis media) using neisserial surface protein A (NspA-men); b) *Neisseria gonorrhoeae* (gonorrhea) using neisserial surface protein A (NspA-gon); c) *Streptococcus pneumoniae* (pneumococcal pneumonia, otitis media, meningitis) using other pneumococcal protein antigen; d) vaccines against *Streptococcus equi* ("strangles" in horses) using a *Streptococcus equi* surface protein; e) vaccines against influenza virus *helicobacter pylori* (gastric ulcer), respiratory pathogens including *Pseudomonas aeruginosa*, f) contraceptive vaccines using zona pellucida antigens; g) vaccine against respiratory syncytial virus; h) generation of "oral tolerance" to auto-antigens (auto-immune conditions); i) vaccines against mycoplasma infections; and j) vaccines against *Staphylococcus aureus* protein A.

In yet another embodiment, the vaccine construction technology of the present invention can be used to generate immunity mediated by so-called cytotoxic T cells instead of antibodies. This methodology would have applications especially against viral infections.

Thus, the present invention is directed to a plasmid capable of replication in a host which comprises, in operable linkage:

a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin. In addition, the plasmid may further comprise DNA sequences encoding subunit B of cholera toxin fused to the A2 subunit of cholera toxin. One such preferred plasmid is pCT$^{\Delta A1}$ (deposited with ATCC, 10801 University Blvd., Manassas, Va. 20110-2209 on May 4, 1999, designation PTA-4). In another embodiment, the plasmid further comprises salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused to the A2 subunit of cholera toxin. One such preferred plasmid is designated pSBR-CTA2/B or pSBR-CT$^{\Delta A1}$ (deposited with ATCC, 10801 University Blvd., Manassas, Va. 20110-2209 on May 4, 1999, designation PTA-5).

In another embodiment, the present invention is directed to a plasmid capable of replication in a host which comprises, in operable linkage: a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin, further comprising DNA sequences encoding an antigen of interest fused to DNA sequences encoding the A2 subunit of cholera toxin. In addition, there is provided a capable of replication in a host which comprises, in operable linkage: a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin further comprising DNA sequences encoding an antigen of interest fused to DNA sequences encoding the A2 subunit of cholera toxin. This plasmid may further comprises salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused to the A2 subunit of cholera toxin. The present invention also relates to chimeric proteins and fusion proteins produced by the plasmids of the present invention.

In another embodiment, the present invention is directed to an attenuated bacterial strain containing a plasmid of the present invention. In a preferred embodiment, the bacterial strain is Salmonella.

In another embodiment, the present invention is directed to a method of producing an immune response to a protein antigen of interest in an individual in need of such treatment, comprising the step of administereing to said individual a pharmacologically effective dose of a chimeric protein of the present invention. The protein may be administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, and subcutaneously. Preferably, the immune response results in the production of antibodies to the protein antigen sequence in a bodily fluid selected from the group consisting of saliva, intestinal secretions, respiratory secretions, genital secretions, tears, milk and blood. Preferably, the immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues of said individual, the development of cytotoxic T cells and immunological tolerance to the protein antigen sequence.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

One object of the present invention was to demonstrate the duration of antibody responses to the AgI/II adhesin after oral immunization of mice with SBR-CT$^{\Delta A1}$ about 1 year earlier. One group consisted of five mice previously given three doses of 100 μg of SBR-CT$^{\Delta A1}$ together with 5 μg of intact CT as an adjuvant (except for animal #5 in FIGS. 1A/1C and FIGS. 2A/2C, the mice were given the dose adsorbed on $Al(OH)_3$, which was shown to enhance serum IgG antibody responses after oral immunization).

A second group comprised three similarly treated mice with the exception that they were immunized in the absence of intact CT. A third group consisting of six mice which were sham immunized with buffer only were used as naive controls. Saliva and serum samples were collected 11 months after the last dose of the primary immunization and all three groups of mice were subsequently given 100 μg of SBR-CT$^{\Delta A1}$ by gastric intubation. CT adjuvant (5 μg) was co-administered to those mice that had also received CT during the primary immunization, and to half of the naive control animals. Samples of saliva and serum were collected again 7 days after the booster immunization and antibody responses were evaluated by ELISA on plates coated with native AgI/II and CT. Unknown antibody concentrations were calibrated against mouse immunoglobulin reference serum standards assayed simultaneously in the same microtiter plate. Results were evaluated by Student's t test using the MultiStat program (Biosoft, Cambridge, UK) with a Macintosh computer. Differences were considered significant at the $P<0.05$ level.

Substantial levels of serum IgG (FIGS. 1A and 1B) and salivary IgA (FIGS. 2A and 2B) antibodies to AgI/II and CT persisted at least until day 357, although lower than immediately after immunization (day 28), even in mice that did not receive an adjuvant dose of intact CT (FIGS. 1B and 1D plus FIGS. 2B and 2D). During the same period, the six sham-immunized mice did not develop detectable serum or salivary antibody responses, except two animals that showed trace levels of salivary IgA to AgI/II (0.15 and 0.12 % antibody/total IgA). However, the response of the sham-immunized group (0.05±0.07% antibody/total IgA) was significantly less ($P<0.05$) than the salivary responses in either of the immunized groups (0.82±0.56% antibody/total IgA (FIG. 2A) and 0.51±0.27% antibody/total IgA (FIG. 2B)). The prolonged duration of antibody responses might be explained by persisting antigen providing continuous low-level stimulation of memory cells. The mechanism of antigen persistence may involve follicular dendritic cells which bind antigen-antibody complexes via cell surface Fc receptors and slowly release them over long periods. Alternatively, the existence of molecules cross-reacting with AgI/II (or cross-reactive enterotoxins in the case of CT) cannot be ruled out, although *S. mutans* is not a natural inhabitant of the murine oral cavity.

A recall response was not observed in serum after the oral booster immunization (FIGS. 1A and 1C), as the antibody responses to AgI/II and CT before and immediately after the booster immunization were not significantly different. However one mouse (#3 in FIG. 1C) that had the lowest antibody levels to CT showed a remarkable 16-fold increase resulting in a higher final response than was observed shortly after the primary immunization. This mouse also showed an enhanced anamnestic IgG response to AgI/II which was 13 times higher than observed immediately prior to the boost (FIG. 1A). This finding suggests that anamnestic responses are not readily elicited in the presence of a relatively high persisting antibody response. As expected, naive mice developed a poor IgG antibody response to AgI/II or CT upon challenge with one dose of SBR-CT$^{\Delta A1}$.

An enhanced salivary IgA anamnestic response was not observed in these mice following the oral booster immunization, even when CT was used as an adjuvant (FIGS. 2A and 2C). Trace levels of salivary antibody to AgI/II observed in two of the six naive mice were not altered after the single booster immunization, while the remaining mice did not develop any salivary response. It appears that, after peroral immunization, the anamnestic response in the salivary glands may depend on recruitment of memory cells from the Peyer's patches or other mucosal induction sites, whereas the gut lamina propria may possess an additional source of memory represented by local memory cells that differentiate into plasma cells upon in situ activation by antigen adsorbed through intestinal epithelial cells. This might result in the memory response being manifested more readily at the gut lamina propria than at a remote effector site such as the salivary glands.

SBR represents an AgI/II adherence domain that mediates the binding of *S. mutans* to the saliva-coated tooth surfaces. S-IgA antibodies to the whole AgI/II molecule inhibit *S. mutans* adherence in vitro as well as *S. mutans* colonization and dental caries development in vivo. Since *S. mutans* infects more than 95% of the human population and caries is a common infectious disease, the continuous presence of salivary S-IgA as well as serum-derived IgG antibodies may be necessary to suppress an organism that is continually present in the oral cavity. The present invention shows that induction of long-term antibody responses is possible upon primary immunization with the SBR-CT$^{\Delta A1}$ chimeric protein. This is further supported by the finding that AgI/II-responsive T cells persist in cervical and mesenteric lymph nodes for now up to eleven months after immunization. This immunization strategy applied to other mucosal infections by linking candidate immunogens to CT$^{\Delta A1}$, may similarly elicit prolonged mucosal antibody responses.

EXAMPLE 2

Strain Construction

SBR-CT$^{\Delta A1}$ was expressed in *S. typhimurium* BRD509, an aroA$^-$ aroD$^-$ oral vaccine strain after electrotransformation with plasmids pSBR-CT$^{\Delta A1}$ and pGP-1-2 using a gene pulser (Biorad, Richmond, Calif.) set at 2.5 kV, 25 μF, and 200 Ohms. The for mer plasmid expresses SBR-CT$^{\Delta A1}$ under the inducible control of the bacteriophage T7 promoter, while the latter provides a source of T7 RNA polymerase that is temperature-regulated. Specifically, the T7 RNA polymerase is under the control of the 1 P_L promoter that is regulated by the cI857 temperature-sensitive 1 repressor. Colonies transformed with both plasmids were selected on L-agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% dextrose, 1.5% agar) supplemented with 50 μg/ml carbenicillin plus 50 μg/ml kanamycin (to select for pSBR-CT$^{\Delta A1}$ and pGP1-2, respectively). Transformants were examined for the presence of plasmids with sizes of 5.6 and 7.2 kilobases, corresponding to the size of pSBR-CT$^{\Delta A1}$ and pGP1-2, respectively.

EXAMPLE 3

Target Protein Expression and Localization

Colonies positive for both pGP1-2 and pSBR-CT$^{\Delta A1}$ were grown at 30° C. in L-broth containing the appropriate

antibiotics and target gene expression was induced at mid-log phase by a temperature shift to 42° C. After 30 minutes the cultures were returned to 30° C. and incubation was continued for an additional 90 minutes. To determine expression of SBR-CT$^{\Delta A1}$, whole-cell lysates were examined by G_{M1}-ELISA for the presence of a G_{M1} ganglioside-binding soluble protein that would react with polyclonal antibodies to CTB or AgI/II, or with a monoclonal antibody specific for the SBR of AgI/II. The insoluble pellet was then processed and possible inclusion bodies were isolated, solubilized by boiling in sodium dodecyl sulfate (SDS) buffer (the amount used was proportional to the final absorbance at 600 nm of the corresponding cultures), and samples (3 μl) analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel. To determine whether SBR-CT$^{\Delta A1}$ is transported to the periplasm, periplasmic extracts were prepared by either the spheroplast formation method or by cold osmotic shock. The extracts obtained were essentially free of cytoplasmic contamination (≦2%) as determined by assaying the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme. The total protein content of the extracts was estimated by the bicinchoninic acid protein assay method (Pierce, Rockford, Ill.) using bovine serum albumin as the standard.

EXAMPLE 4

Oral Immunization

The bacteria were grown at 30° C. to an optical density at 600 nm of 0.5–0.6, harvested by centrifugation, and resuspended in a medium consisting of 4 parts Hank's balanced salt solution (Life Technologies Inc., Grand Island, N.Y.) and 1 part sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The number of bacteria in the suspension was estimated by extrapolating from a growth curve and was confirmed by plating dilutions on L-agar plates (with or without the appropriate antibiotics) and enumerating the colonies grown after overnight incubation at 30° C. The immunizing dose (10^9 colony-forming units in 0.25 ml) was administered to 10-week old BALB/c mice by intragastric intubation using a 22-gauge feeding tube (Popper and Sons Inc., Hyde Park, N.Y.). The animals were immunized on days 0 and 49 and sampled at weekly or bi-weekly intervals. Serum was obtained from tail vein blood samples and saliva was collected after stimulation of the salivary flow by intraperitoneal injection of 5 μg carbachol. Serum IgG and salivary IgA antibodies were determined by ELISA on microtiter plates coated with AgI/II or G_{M1} ganglioside followed by CT, while total salivary IgA concentrations were assayed on plates coated with antibodies to mouse IgA. Peroxidase-conjugated antibodies to mouse IgG or IgA were used as detection reagents (Southern Biotechnology Associates, Birmingham, Ala.). The amount of antibody/immunoglobulin in test samples was calculated by interpolation on standard curves generated using a mouse immunoglobulin reference serum and constructed by a computer program based on four parameter logistic algorithms (Softmax, Molecular Devices, Menlo Park, Calif.). Results were evaluated by Student's t test and differences were considered significant at the $P<0.05$ level.

EXAMPLE 5

Results

Recombinant *S. typhimurium* BRD509 positive for both pGP1-2 and pSBR-CT$^{\Delta A1}$ was shown to produce a protein that bound the G_{M1}-ganglioside receptor and possessed CTB and AgI/II epitopes, in contrast to the original BRD509 strain or clones containing either pGP1-2 or pSBR-CT$^{\Delta A1}$ alone (TABLE I). Since the pSBR-CT$^{\Delta A1}$ encodes for the signal peptides of CTB and SBR-CTA2, it was of interest to determine whether the chimeric protein was transported into the periplasm where assembly of its components takes place.

TABLE I

G_{M1} Ganglioside-binding activity and antigenicity of soluble protein extracts[a] from recombinant *S. typhimurium* BRD509 clones

	ELISA value[b] of clone extract developed with		
Plasmid	Anti-CTB	Anti-AgI/II	Anti-SBR
None	0.017	0.003	0.001
pGP1-2	0.009	0.017	0.008
pSBR-CT$^{\Delta A1}$	0.02	0.013	0.004
pGP-1-2 + pSBRCT$^{\Delta A1}$	2.052	1.005	1.01

a = assayed at 20 μg total protein/ml; b = mean optical density at 490 nm

To examine this, a calibrated G_{M1}-ELISA standardized with purified SBR-CT$^{\Delta A1}$ was used to detect and quantify the chimeric protein in periplasmic and in whole-cell extracts under uninduced and induced (temperature shift from 30° C. to 37° C. or 42° C. for 30 minute) conditions. As shown in TABLE II, SBR-CT$^{\Delta A1}$ was found in the periplasm of *S. typhimurium* BRD509 (pGP1-2+pSBR-CT$^{\Delta A1}$) and of *E. coli* BL21(DE3) (pSBR-CT$^{\Delta A1}$) which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter. The chimeric protein was not detected in the periplasm or whole-cell lysates of a negative control clone lacking pSBR-CT$^{\Delta A1}$ (TABLE II). Although cellular location of the foreign antigen may affect the immune response, secretion of a protein into the periplasm may enhance its stability by preventing degradation.

TABLE II

Localization of SBR-CT$^{\Delta A1}$ Chimeric Protein in the Periplasmic Space

		Periplasmic fraction obtained by:		
Strain	Induction	Spheroplast formation	Osmotic Shock	Whole Cell lysate
E. coli BL21 (DE3) (pSBR-CT$^{\Delta A1}$)	IPTG[a]	271[b]	319	99.7
S. typ. BRD509 (pGP1-2)	42° C.	0	0	0
S. typ. BRD509 (pGP1-2 + PSBR-CT$^{\Delta A1}$)	None	77.3	98.5	20.2
S. typ. BRD509 (pGP1-2 + pSBR-CT$^{\Delta A1}$)	37° C.	239	314	119
S. typ. BRD509 (pGP1-2 + pSBR-CT$^{\Delta A1}$)	42° C.	119	123	53.6

a = isopropyl-β-D-thiogalactoside
b = μg SBR-CT$^{\Delta A1}$/mg protein in the extracts

Under uninduced conditions, the chimeric protein was produced at about 20 μg per mg of total soluble protein (TABLE II) or 7–9 μg per 10^9 bacteria. This finding is likely due to residual expression of the T7 RNA polymerase. Indeed, the temperature-sensitive I repressor on pGP1-2 does not tightly repress the I P_L promoter which consequently allows low-level production of the polymerase at 30° C. The amount of soluble chimeric protein increased several-fold following induction at 37° C., whereas at 42° C. the increase was minimal (TABLE II) with concomitant

accumulation of SBR-CT$^{\Delta A1}$ in inclusion bodies (FIG. 3). This suggests that at physiological body temperature (36–37° C.) production of soluble chimeric protein may be optimal.

Recombinant *S. typhimurium* expressing SBR-CT$^{\Delta A1}$ was routinely grown under uninduced conditions (30° C.) at which it showed optimal growth. Although the SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* strain does not possess a temperature-sensitive mutation, temperatures higher than 30° C. cause gradual induction of the highly-efficient T7 RNA polymerase which may interfere with gene transcription by the host RNA polymerase. Additionally overexpression of the cloned chimeric protein at 37° C. could interfere with bacterial growth. The strain was also found to be immunogenic since oral administration of 10^9 bacteria in mice resulted in serum IgG and salivary IgA antibody responses to Salmonella and native AgI/II and CT (FIGS. 4A and 4B). An oral booster immunization 7 weeks later significantly enhanced the serum IgG response to AgI/II and CT ($P<0.05$) but not to Salmonella, the response to which reached high levels even before the secondary immunization (FIG. 4A). Salivary IgA antibodies to Salmonella and the cloned antigens were detected 3 weeks after the primary immunization (FIG. 4B). The salivary response to AgI/II approached the level of 1% specific IgA antibody/total IgA but was not enhanced after the booster immunization. This is in contrast to the response against CT or Salmonella, where the increase in the response to CT or Salmonella reached statistical significance at weeks 8 or 10, respectively.

The salivary IgA response to AgI/II (~1% antibody/total IgA) after a single oral immunization with Salmonella is similar to that observed after 3 doses of 100 μg purified SBR-CT$^{\Delta A1}$ in the absence of intact CT adjuvant. The immunizing dose (10^9 bacteria) was estimated to contain 7–9 μg of chimeric protein, but this probably does not reflect the amount of SBR-CT$^{\Delta A1}$ delivered in vivo, which would largely depend on the extent of tissue colonization by Salmonella.

In the present invention, the SBR-CT$^{\Delta A1}$ chimeric protein was expressed in attenuated *S. typhimurium* and oral immunization with this recombinant strain resulted in serum IgG and salivary IgA antibody responses against Salmonella and the cloned antigens. Since the SBR segment of AgI/II plays an important role in *S. mutans* colonization, salivary IgA antibodies to SBR may confer protection against this oral pathogen.

EXAMPLE 6

Antigens

AgI/II was purified chromatographically from the culture supernatant of *S. mutans* essentially as described by Russell, et al., 1980, 28:486–493. The SBR-CTA2/B chimeric protein was constructed and expressed in *E. coli* and purified from extracts. In essence, this consisted of PCR-amplifying DNA for a 42-kDa segment encompassing the A-repeat region and some downstream sequence of AgI/II from the pac gene, ligating this in a modified pET20b(+) plasmid (Novagen, Inc., Madison Wis.) in frame with and upstream of the genes for CTA2 and CTB, and transforming the recombinant plasmid into *E. coli* BL21(DE3) cells (Novagen).

SBR polypeptide was obtained by excising the relevant DNA and religating it into unmodified pET20b(+) in order to express SBR with a 6-residue histidine sequence derived from the plasmid. This plasmid was also expressed in *E. coli* BL21(DE3), and SBR was purified from cell lysates by metal-chelation chromatography on a nickel-loaded column (Novagen), according to the manufacturer's instructions. CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell Calif.).

EXAMPLE 7

Animals and Immunization

Adult BALB/c mice of either sex, 14 to 20 weeks old, from a pathogen-free colony, were used for all experiments. Groups of 9 mice were immunized i.g. 3 times at 10-day intervals by gastric intubation of either SBR-CTA2/B (100 μg) alone, SBR-CTA2/B together with 5 μg of CT as an adjuvant, or an equimolar amount of SBR (40 μg), all given in 0.5 ml of 0.35M $NaHCO_3$. Serum and saliva samples were collected on day 0 and 10 days after each immunization for assay of antibodies by ELISA. In some experiments, subgroups of 3 mice were killed 10 days after each immunization, for the preparation of cells from PP, MLN, and spleens for T cell proliferation and flow cytometric analyses.

EXAMPLE 8

ELISA

Serum IgG and salivary IgA antibodies to AgI/II, and total salivary IgA concentrations were determined by ELISA, as described by Russell, et al., 1991, *Infect. Immun.* 59:4061–4070, on plates coated with AgI/II or anti-mouse IgA, respectively, and using goat anti-mouse IgG and IgA peroxidase conjugates as detection reagents (Southern Biotechnology Associates, Inc., Birmingham Ala.). Unknowns were interpolated on calibration curves constructed by a computer program based on four parameter logistic algorithms.

EXAMPLE 9

Preparation and Culture of Lymphoid Cells

Single-cell suspensions were obtained by teasing PP, MLN, and spleen apart with needles, and tissue debris was removed by filtering through nylon mesh. Peripheral blood mononuclear cells were obtained by centrifugation on Histopaque 1083 (Sigma Diagnostic, St. Louis, Mo.). Remaining erythrocytes were lysed in buffered ammonium chloride, the cells were washed thrice in RPMI 1640 (Cellgro) medium supplemented with 2% fetal calf serum (FCS), and were finally resuspended 10% FCS/RPMI 1640. Cells were cultured in 10% FCS/RPMI 1640 supplemented with 1 mM sodium pyruvate (Cellgro), non-essential amino acids (Cellgro), 2 mM glutamine (Cellgro), 100 U/ml penicillin-streptomycin (Cellgro), 25 mM HEPES (Sigma), and 0.01 mM 2-mercaptoethanol (Sigma).

EXAMPLE 10

Flow Cytometry

Cell marker expression on freshly isolated cells was determined by double-staining with biotinylated anti-CD4 (GK1.5) followed by avidin-phycoerythrin, and either FITC-conjugated anti-CD3 (145-2C11) or FITC-conjugated anti-CD8 (53-6.72), by incubating for 30 minutes at 4° C. in 2% FCS/Dulbecco's PBS with 0.02% NaN_3. Cells were washed, fixed in 1% paraformaldehyde overnight, and analysed on a FACStar IV flow cytometer (Becton-Dickinson).

EXAMPLE 11

Proliferation Assay

Cells from PP, MLN, and spleen were incubated at 10^5 cells/well (0.1 ml) in triplicate with a previously optimized concentration of AgI/II (0.5 μg/ml) for 5 days, and were pulsed with ^{3}[H]-thymidine (0.5 μCi/well) 8 hours before

harvesting. Uptake of ^{3}[H] was counted by liquid scintillation counter. The stimulation index was calculated as: cpm (wells with AgI/II)/mean cpm (control wells).

EXAMPLE 12

Cytokine Expression

The expression of cytokines by PP, MLN, and spleen cells after culture in vitro with or without AgI/II (0.1 μg/ml) for 24 hours was determined by a reverse-transcription and polymerase chain-reaction (RT-PCR) procedure for the amplification of cytokine mRNA. Cells (5–7×10^6) were harvested from the cultures, washed thoroughly, and then lysed in 350 μl of lysing buffer for isolation of RNA using the RNeasy kit (Qiagen Inc., Chatsworth Calif.). RNA was redissolved in 40 μl of diethyl pyrocarbonate-treated water, and 2 μl samples were added to 18 μl of RT mixture (Perkin-Elmer, Foster City Calif.) containing 1× PCR buffer, 5 mM $MgCl_2$, 1 mM of each deoxyribonucleoside triphosphate, 1 U/ml RNase inhibitor, 2.5 U/ml Moloney murine leukemia virus reverse transcriptase, and 2.5 mM Oligo $d(T)_{16}$. Mixtures were overlaid with 50 μl of light mineral oil and incubated in a thermal cycler (Perkin-Elmer) for 15 minutes at 42° C., 45 minutes at 37° C., 5 minutes at 99° C., and 5 minutes at 4° C. After reverse transcription, 80 μl of PCR mixture (Perkin-Elmer) was added to each tube to give final concentrations of 25 U/ml AmpliTaq DNA polymerase, 0.15 μM 5' primer, 0.15 μM 3' primer, 2 mM $MgCl_2$, and 1× PCR buffer II. Primers specific for murine IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and β-actin were obtained from Clontech Laboratories Inc. (Palo Alto, Calif.) or the Oligonucleotide Synthesis Core Facility of the UAB Comprehensive Cancer Center, and their specificity was verified by means of RT-PCR on RNA extracted from mitogen-stimulated mouse spleen cells. After heating at 95° C. for 2 min, cDNA was amplified for 35 cycles consisting of: 45s at 94° C., 3 minutes at 72° C., and 2 minutes at 60° C. The products of amplification were analysed by 2% agarose gel electrophoresis, revealed by ethidium bromide staining, and photographed by UV transillumination. The results were scored according to the presence of a band of appropriate molecular size: –, no detectable band; ±, very faint or uncertain band; +, clearly detectable band; ++, very strong band.

EXAMPLE 13

Statistical Methods

Quantitative results were evaluated by Student's t test, by means of MultiStat (Biosoft, Ferguson Mo.) on a Macintosh computer. Antibody data were transformed logarithmically to normalize their distribution and homogenize the variances.

EXAMPLE 14

Antibody Responses

I.g. immunization of mice with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT incrementally induced serum IgG and salivary IgA antibodies measured against whole AgI/II (FIGS. 5A and 5B). Immunization with SBR alone resulted in weak but statistically significant ($P<0.001$ at all intervals) serum IgG antibody responses, and modest salivary IgA antibodies that were significantly elevated above background only after the second and third immunization ($P<0.001$ and $P<0.01$, respectively). Administration of the SBR-CTA2/B chimeric protein generated significantly greater serum IgG responses ($P<0.001$), and co-administration of CT as an adjuvant further enhanced both the level and the earlier development of serum IgG antibodies. Salivary IgA antibodies also tended to be elevated by immunization with SBR-CTA2/B chimeric protein especially when given with CT as adjuvant. However, because of variation between animals, statistical significance was attained only after 2 doses given with CT. Nevertheless, the general pattern of results was in accordance with expectations based on responses to AgI/II alone or chemically conjugated to CTB, and administered i.g. without or with CT adjuvant. Total salivary IgA concentrations also increased in all animals during the immunization period, from 2.13±0.61 μg/ml in unimmunized animals to 5.92±0.64 μg/ml after 3 immunizations, but there were no significant differences between the immunization groups.

EXAMPLE 15

T Cell Proliferative Responses

To show whether T cells capable of proliferating in vitro in response to stimulation with AgI/II had been induced by the first, second, or third i.g. dose, groups of 3 mice were killed 10 days after a first, second, or third immunization with each immunogen preparation, and mononuclear cells from PP, MLN, and spleens were cultured with or without AgI/II. Incorporation of ^{3}[H]-thymidine expressed as stimulation indices revealed that AgI/II responsive cells were elicited in the lymphoid tissues associated with the intestine, incrementally with the number and form of the immunogen doses (FIG. 6). PP and MLN cells taken from mice given 2 or 3 doses of SBR or of SBR-CTA2/B alone showed modest proliferative responses to AgI/II in vitro (stimulation indices in the range 2.4–3.2; 5.44 for PP from mice given 3 doses of SBR-CTA2/B), whereas PP and MLN cells from mice immunized with SBR-CTA2/B plus CT adjuvant showed proliferative responses after one dose (stimulation indices 2.3 and 3.6, respectively), and greater responses after 2 or 3 doses (stimulation indices 3.1–6.1). The proliferative responses of PP and MLN cells were different: MLN cells responded similarly to (or less than) PP cells when taken from mice immunized with SBR or SBR-CTA2/B, but showed greater responses to AgI/II in vitro when taken from mice given AgI/II-CTA2/B plus CT. Spleen cells generally did not respond to stimulation with AgI/II in vitro (stimulation indices <2), except for those taken from mice immunized once with SBR-CTA2/B plus CT (stimulation index=2.8). Cells from the PP, MLN, or spleens of unimmunized mice did not proliferate in response to AgI/II in vitro (stimulation indices 1.2–1.5).

EXAMPLE 16

T Cell Surface Marker Analysis

To elucidate the nature of the T cell responses to i.g. immunization, cells freshly isolated from PP, MLN, spleen, or peripheral blood of mice immunized once, twice, or three times with the different immunogens were analyzed by flow cytometry for the proportion of cells expressing T cell markers CD3 (all T cells), CD4 (T helper phenotype), or CD8 (T suppressor/cytotoxic phenotype). The results are shown in FIG. 7. Among PP cells, there was an increase in the proportion of total T cells after each immunization that was most noticeable in animals immunized with SBR-CTA2/B or SBR-CTA2/B plus CT. This increase was mostly in the CD4$^+$ T helper population, whereas the CD8$^+$ T suppressor/cytotoxic population remained small. The MLN cell populations remained more stable, except in the case of cells from mice immunized with SBR-CTA2/B plus CT in which the CD4$^+$ population increased with the number of immunizations. MLN generally, however, contained more T cells of both phenotypes than PP, regardless of immunization status. Peripheral blood cells tended to show the greatest increases in the proportion of CD4$^+$ T cells after

immunization, especially with SBR-CTA2/B plus CT, although these numbers must be interpreted with caution because of the small numbers of cells obtained. Spleen cells showed modest increases in the proportions of CD4+ T cells after immunization in all groups.

alone revealed type 1 (IFN-γ and IL-2) as well as type 2 (IL-4) cytokine responses upon stimulation in vitro, whereas cells from the same organs of mice immunized with SBR-CTA2/B chimeric protein revealed IL-4 but little or no type 1 cytokine response.

TABLE III

Cytokine expression in PP, MLN and spleen cell cultures of mice immunized with SBR, SBR-CTA2/B or SBR-CTA2/B + CT

		IFN-γ			IL-2			IL-4			IL-5		
Immunization[a]	Culture[b]	P	M	S	P	M	S	P	M	S	P	M	S
SBR	Control	–[c]	–	–	–	±	–	±	–	–	+	+	+
	+AgI/II	+	+	+	++	+	++	++	–	++	+	+	+
SBR-CTA2/B	Control	–	–	–	–	±	–	+	±	+	+	+	+
	+AgI/II	–	±	+	–	+	++	+	+	++	+	+	+
SBR-CTA2/B + CT	Control	–	–	+	–	±	+	±	±	+	+	+	+
	+AgI/II	–	+	++	–	±	++	+	+	++	+	+	+

[a]mice were immunized thrice at 10 day intervals and organs collected 3 days after the last immunization.
[b]cells were cultured in vitro for 24 hours without (control) and with AgI/II (0.1 ug/ml)
[c]cytokine mRNA expression detected by RT-PCR and scored according to the presence of ethidium bromide-stained band of appropriate molecular size;
–; no detectable band
±; very faint/uncertain band;
+, clear band; and
++, very strong band.
P = PP cell cultures,
M = MLN cell cultures;
S = spleen cell cultures.

EXAMPLE 17

Cytokine Expression

To elucidate the pattern of expression of cytokines, PP, MLN, and spleen cells were taken from mice immunized three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT 3 days after the last dose, cultured in vitro for 24 hours with or without AgI/II, and examined for the presence of mRNA for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 by RT-PCR. After culture with AgI/II, PP, MLN, and spleen cells from mice immunized with SBR alone revealed mRNA for IFN-γ and IL-2, but only PP and spleen cells also revealed IL-4 mRNA, whereas IL-5 mRNA was detectable in all cell cultures regardless of stimulation (TABLE III). PP cells from mice immunized with SBR-CTA2/B, without or with CT adjuvant, did not reveal mRNA for IFN-γ or IL-2, even after culture with AgI/II, and MLN cells from these animals revealed variable IFN-γ and IL-2 mRNA responses. However, PP, MLN, and spleen cells revealed IL-4 mRNA particularly after stimulation with AgI/II, whereas all cultures were positive for IL-5 mRNA. Likewise, mRNA for IL-6 and IL-10 was found in all cell cultures, regardless of immunization or in vitro stimulation. There was an increase in IFN-γ and IL-2 expression (in response to stimulation with AgI/II in vitro) in PP, MLN, and spleen cells from mice immunized 3 times with SBR alone relative to cells from mice immunized twice. Likewise, spleen cells from mice immunized 3 times with SBR-CTA2/B (without or with CT) showed increased AgI/II-specific expression of IFN-γ, IL-2, and IL-4 relative to twice-immunized mice. Cells from unimmunized mice did not respond in culture with AgI/II by the expression of IFN-γ, IL-2, and IL-4 mRNA above that revealed in control cultures, except that spleen cells showed weak evidence of IFN-γ expression on culture with AgI/II. Thus, PP and MLN cells from mice immunized with SBR

Lymphocytes taken from the PP and MLN of mice immunized i.g. with SBR, or SBR-CTA2/B without or with CT as adjuvant were capable of proliferating in vitro when stimulated with AgI/II, showing a similar overall pattern of T cell responses to the different regimens and stages of immunization as the serum and salivary antibody responses. Immunization with SBR alone induced the lowest proliferative responses in PP and MLN cells, and this was reflected also in little change in the proportions of CD4+ and CD8+ T cells in these organs. Moreover, the pattern of cytokine expression in the cells from PP and MLN of these mice suggested a mixed type 1 and type 2 helper activity, possibly governed by Th0 cells. Coupling SBR to CTB in the form of the SBR-CTA2/B chimeric protein enhanced its immunogenicity with respect to T cell responses in PP and MLN, and the addition of CT as an adjuvant further elevated these responses. Furthermore, the cytokine expression pattern in PP and MLN cells from mice immunized with SBR-CTA2/B (with or without CT) indicated that T cell help was skewed towards Th2 activity. However, the finding of IL-5, IL-6, and IL-10 mRNA in cell cultures regardless of antigen stimulation in vitro is not readily explained in these terms, but may indicate constitutive expression of these cytokines or their continued expression ex vivo after immunization. It is also possible that Il-6 and IL-10 mRNA were derived from macrophages present in the cell cultures, although these would be largely adherent and unlikely to be harvested along with the lymphocytes.

The proportions of CD4+ T cells in PP increased after each additional dose of these immunogen preparations, but a corresponding increase was seen in MLN cells only from mice immunized with SBR-CTA2/B chimeric protein and CT adjuvant. The finding that these T cell responses occurred in PP and MLN as early as after the first immunization, at least with SBR-CTA2/B, showed that antigen-sensitized T cells were elicited before IgA antibody responses became elevated in the effector sites of mucosal

immunity such as salivary glands. The responses in MLN and PP were different, as significant proliferative responses and increased proportions of $CD4^+$ cells during the course of immunization were developed in MLN cells only when CT was used as an adjuvant, and moreover MLN from all mice contained higher proportions of T cells of both phenotypes than corresponding PP. The proportion of $CD8^+$ cells was higher in MLN than in PP, but as it was not reduced by the administration of CT as an adjuvant, it appears that the enhanced AgI/II-specific proliferation in MLN cells from mice given CT is not due to inhibition of $CD8^+$ suppressor cells by CT. The spleen, a non-mucosal lymphoid organ, displayed little or no response in terms of antigen-specific proliferating T cells, despite the considerable elevation of serum IgG antibodies especially when SBR-CTA2/B was given together with CT adjuvant. This is consistent with the relatively modest numbers of specific antibody-secreting cells found in the spleen after i.g. immunization with AgI/II chemically conjugated to CTB and given with CT. Throughout these experiments, although the mice were immunized with SBR or SBR-CTA2/B chimeric protein representing residues 186 to 577 of AgI/II, both antibody and T cell responses could be detected with intact AgI/II. This implies that SBR retains conformational structure similar to that of the corresponding part of the whole AgI/II molecule, and that both are processed similarly by antigen-presenting cells.

These responses are in accordance with the concept of the common mucosal immune system, and the dissemination of antigen-sensitized T and B cells from the inductive sites such as PP, through the MLN that drain the lymph flow from the small intestine, and thence into the circulation prior to relocating in the effector sites of mucosal immunity, including the salivary glands. Thus, i.g. immunization with SBR, especially when coupled to CTB in the form of a chimeric protein, leads to the appearance of antigen-responsive T cells in both PP and MLN. Because few cells were recoverable from blood, it was not practically possible to trace the appearance of such cells in the circulation, although this has been well documented in humans. The transient circulation of specific antibody-secreting cells, predominantly of the IgA isotype, approximately one week after mucosal immunization has been demonstrated in human and animal systems. Curiously, perhaps, it appears that the peak of circulating antigen-specific T cells occurs after the peak of circulating antibody-secreting cells, and an increased proportion of $CD4^+$ T helper cells was found in the peripheral blood of mice 10 days after the second or third dose of SBR-CTA2/B, especially if CT was also given as an adjuvant. Cytokine-secreting T cells occur in effector sites of mucosal immunity, such as the salivary glands.

CT has been shown to enhance T helper responses in intestinal tissues, and particularly the Th2 subset that is held to promote high levels of serum IgG and mucosal IgA antibody responses. Type 2 cytokine production by antigen-specific T cells in nasal-associated lymphoid tissue and the draining cervical lymph nodes of mice immunized intranasally, as well as in PP and MLN of mice immunized i.g., with AgI/II conjugated to CTB were also found. CT is known to deplete selectively $CD8^+$ intraepithelial lymphocytes and while the functions and migratory potential of these cells are incompletely understood, any such effect within inductive sites such as the PP would also serve to elevate the proportion of $CD4^+$ T cells. However, although the proportion of $CD8^+$ cells declined slightly in some tissues, this appeared to occur concomitantly with an increase in the number of $CD3^+$ cells, in particular the $CD4^+$ subset. Whether CTB itself can serve as an adjuvant in the absence of intact CT has been controversial. Synergism between CTB and CT has been demonstrated and most commercially available, non-recombinant preparations of CTB contain small amounts of intact CT that may be sufficient to show this effect. The genetically constructed SBR-CTA2/B chimeric protein, in which the toxic CTA1 subunit has been deleted, is clearly able to induce mucosal and circulating antibodies without the necessity for additional CT. The adjuvant activity of CT may be closely linked to its toxicity which is a function the ADP-ribosyltransferase activity of the A1 subunit. Adjuvanticity of the related *Escherichia coli* heat-labile enterotoxin can be dissociated from toxicity. Fusion proteins of CTB directly coupled to other antigenic peptides have been constructed, but the conformation of CTB and its ability to form G_{M1}-binding pentamers tend to be disrupted by peptides longer than approximately 12 amino acid residues and moreover, their mucosal immunogenicity seems to be limited in the absence of additional CT. These limitations do not apply to SBR-CTA2/B chimeric protein, in which a large 42 kDa segment of protein is fused to the CTA2 subunit which couples it noncovalently to the CTB pentamer to preserve its G_{M1} ganglioside-binding activity. The enhanced enteric immunogenicity of SBR-CTA2/B chimeric protein, even in the absence of CT, is advantageous for an oral vaccine, as recombinant CTB has been shown to be a safe and effective immunogen in humans.

I.g. immunization with SBR, especially when genetically coupled to CTB to enhance both mucosal and circulating antibody responses, induces T cell responses in the gut-associated lymphoid tissues such as PP and MLN. Furthermore, these T cell responses occur after one or two doses of immunogen, earlier than the antibody responses, and include increased proportions of $CD4^+$ T helper cells. The responses are enhanced by, but are not dependent upon, the addition of CT as an adjuvant.

EXAMPLE 18

Expression of SBR in *S. typhimurium*

The pac gene segment encoding the SBR region (1.2 kilobases [kb]) was removed from pSBR-CT$^{\Delta A1}$ by restriction digestion with the NcoI and XhoI endonucleases, and purified after agarose gel electrophoresis of the digest and extraction using the QIAEX gel extraction kit (Qiagen, Chatsworth, Calif.). The pET20b(+) expression vector (3.7 kb; Novagen, Madison, Wis.) was similarly digested by NcoI and XhoI, dephosphorylated by calf intestinal alkaline phosphatase and purified by gel extraction. The pac segment was then ligated into pET20b(+) in frame with the 3' end of the pelB leader sequence (required for the transport of cloned polypeptides into the periplasm) and the resulting ligation product, designated pSBR, was introduced into *S. typhimurium* BRD509 (pGP1-2) (6) by means of electroporation using a gene pulser (Biorad, Richmond, Calif.) set at 2.5 kV, 25 μF, and 200 Ohms. Transformed colonies were selected on L-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% dextrose, 1.5% agar) supplemented with 50 μg/ml carbenicillin and 50 μg/ml kanamycin (to select for pSBR and pGP1-2, respectively). Transformants were examined for the presence of two plasmids with sizes of 4.9 and 7.2 kb, corresponding to the size of pSBR and pGP1-2, respectively. Both plasmids were required for the expression of SBR since its transcription in pSBR is under the control of the bacteriophage T7 promoter and pGP1-2 provides a source of T7 RNA polymerase. Expression of the SBR polypeptide in transformants containing both plasmids was induced at mid-log phase by a shift from 30° C.–37° C., and production of SBR was confirmed by western immunoblot-

ting of cell lysates using antibodies to the native AgI/II molecule (FIG. 8B). Target gene induction in this system is temperature-regulated because the T7 RNA polymerase is under the control of the 1 P_L promoter that is regulated by the cI857 temperature-sensitive 1 repressor (FIG. 8A).

EXAMPLE 19

Estimation of Recombinant Protein Production

To determine the amount of the SBR polypeptide produced by *S. typhimurium* (pGP1-2+pSBR-CT$^{\Delta A1}$) (6) and *S. typhimurium* (pGP1-2+pSBR) (FIG. 8B), a calibrated "sandwich" ELISA standardized with purified rSBR was performed using cell lysates obtained by sonication. This quantitative ELISA was repeated four times using independent cultures which were grown at 30° C. and subsequently processed for the immunizations (see "Immunizations" below). The construction of standard curves and the interpolation of the unknowns was performed by means of a computer program based on four-parameter logistic algorithms (Softmax/Molecular Devices, Menlo Park, Calif.). For the ELISA, rabbit anti-mouse IgG followed by a mouse monoclonal IgG antibody to SBR served as the coating reagents while peroxidase-conjugated rabbit polyclonal antibodies to native AgI/II was used for detection of bound protein. SBR used as standard was purified from cell lysates by metal-chelation chromatography on a nickel-charged column (Novagen), according to the manufacturer's instructions. The affinity of SBR for nickel arises from a 6-residue histidine sequence (at its C-terminal end) which was derived from the pET-20b(+) expression vector. Recovery of SBR from the column was achieved by elution with imidazole. The purity of the SBR preparation was verified by SDS-PAGE and its protein content was estimated by the bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.) using BSA.

A similar approach was used to quantify the SBR-CT$^{\Delta A1}$ chimeric protein with the exception that the plates were coated with G_{M1} ganglioside (Calbiochem, La Jolla, Calif.). This G_{M1}-ELISA was standardized with SBR-CT$^{\Delta A1}$ purified. Briefly, the chimeric protein was isolated from whole-cell extracts by size-exclusion chromatography on a Superose 12 HR 16/50 column (Pharmacia-LKB, Piscataway, N.J.) followed by anion-exchange chromatography on a Mono Q column (Pharmacia-LKB). Results are expressed as %SBR or SBR-CT$^{\Delta A1}$ per total soluble protein in the lysates.

EXAMPLE 20

Immunizations

Overnight cultures of recombinant *S. typhimurium* BRD509 expressing SBR-CT$^{\Delta A1}$ or SBR were diluted 1:100 in L-broth containing 50 μg/ml of kanamycin and 50 μg/ml of carbenicillin and grown at 30° C. with shaking and aeration until A_{600nm} reached 0.5–0.55. The bacteria were recovered by centrifugation and resuspended in a medium consisting of 4 parts Hank's balanced salt solution (Life Technologies Inc., Grand Island, N.Y.) and 1 part sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The number of bacteria in the suspension was estimated by extrapolating from a growth curve and was confirmed by plating dilutions of the bacterial inoculum on L-agar plates (with or without the appropriate antibiotics) and enumerating the colonies grown after overnight incubation at 30° C.

BALB/c mice, 10 to 12 weeks old, from a pathogen-free colony, were used for oral and i.n. immunization studies performed according to NIH guidelines and protocols approved by the UAB Institutional Animal Care and Use Committee. An oral dose containing 10^9 CFU in 0.25 ml was administered to groups of 5–6 mice by intragastric (i.g.) intubation using a 22-gauge feeding tube (Popper and Sons Inc., Hyde Park, N.Y.) The mice were immunized 1 to 3 times in a period of 6 days. A single booster immunization with 10^{10} CFU was given 15 weeks later. For i.n. immunizations, groups of 6 mice were inoculated 3 times (in a period of 6 days) with 10^8 CFU in a volume of ~20 μl which was slowly applied in the external nares by means of a micropipettor. A single booster i.n. immunization with 10^9 CFU was performed 15 weeks later. In another experiment, groups of 3 mice received a single primary immunization (10^{10} CFU for i.g. or 10^9 CFU for i.n. delivery) followed by a booster immunization with the same dose 15 weeks later. An age-matched, unimmunized control group consisting of 5 mice was also included to monitor background antibody levels during the course of the studies.

EXAMPLE 21

Sampling and Quantification of Antibody Responses

Serum was obtained by centrifugation of blood samples collected from the lateral tail vein with heparinized capillary pipettes. Preimmune samples were obtained 1 day before the immunizations and subsequent to immunizations collections were made 3, 5, and 7 weeks later, one day before the booster immunization (week 15), and at biweekly intervals thereafter (weeks 17 and 19). Saliva samples were collected at the same times as serum by means of a pipettor fitted with a plastic tip after stimulation of salivary flow by i.p. injection of 5 μg carbachol (Sigma Chemical Company, St. Louis, Mo.). Fecal extracts were prepared by vortexing 3 fecal pellets from each mouse in 600 μl extraction buffer (PBS containing 0.02 % azide, 1% BSA, 1 mM PMSF, and 5 mM EDTA). The extracts were subsequently centrifuged and the supernatants obtained were assayed for total IgA levels (see below) and were adjusted to contain 100 μg of total IgA per ml ("standardized" fecal extracts) by adding an appropriate volume of extraction buffer.

The levels of isotype-specific antibodies from serum, saliva, or fecal extracts, and total salivary or intestinal IgA were determined by ELISA on microtiter plates coated with native AgI/II (chromatographically purified from *S. mutans* culture supernatants), G_{M1} followed by CT (List Biological Laboratories, Campell, Calif.), formalin-killed cells of *S. typhimurium* BRD509, or goat anti-mouse IgA. The plates were developed with the appropriate peroxidase-conjugated goat anti-mouse Ig isotype (IgG for serum samples and IgA for secretion samples) and o-phenylenediamine substrate with H_2O_2. IgG 1 or IgG2a antibody responses were assayed using peroxidase-conjugated IgG subclass-specific antibodies. All antibodies used for ELISA were purchased from Southern Biotechnology Associates, Inc., Birmingham, Ala. The concentration of antibodies/total Ig in test samples was calculated by interpolation on standard curves generated using a mouse Ig reference serum (ICN Biomedicals, Costa Mesa, Calif.) and constructed by a program based on four parameter logistic algorithms (Softmax/Molecular Devices).

EXAMPLE 22

Statistical Analysis

Results were evaluated by Student's t test by means of the Multistat program (Biosoft, Cambridge, UK) on a Macintosh computer. Differences were considered significant at the $P<0.05$ level. antibody data were logarithmically transformed to normalize their distribution and homogenize the variances. The data were finally retransformed and presented as geometric means ×/÷ SD.

EXAMPLE 23

Recombinant Protein Production by the Salmonella Clones

Using a "sandwich" ELISA calibrated with purified rSBR, it was determined that *S. typhimurium* (pGP1-2+pSBR-CT$^{\Delta A1}$) and *S. typhimurium* (pGP1-2+pSBR) produced similar amounts of the SBR polypeptide (Table IV). Slightly higher levels of SBR were detected in the lysates from the pSBR-containing clone than in the extracts from the clone expressing the SBR-CT$^{\Delta A1}$ chimeric molecule, but the difference was not statistically significant. This might have resulted from the presence of CTA2/B which could sterically interfere with the recognition of the SBR component of the chimeric protein by the antibodies used in the "sandwich" ELISA. As expected, G_{M1}-ELISA calibrated with purified SBR-CT$^{\Delta A1}$ detected a G_{M1}-binding protein possessing SBR epitopes only in lysates from the pSBR-CT$^{\Delta A1}$-containing clone (TABLE IV). The expression level of SBR-CT$^{\Delta A1}$ in the lysates was approximately 2–3% of the total soluble protein (TABLE IV). These results validate the appropriateness of comparing the capacity of these two clones to induce antibody responses to the SBR of AgI/II.

TABLE IV

Production of recombinant protein by *S. typhimurium* (pGP1-2 + pSBR and *S. typhimurium* (pGP1-2 + pSBR-CT$^{\Delta A1}$)clones

	Elisa Method			Protein Amount[a]	
Assay:	Coating	Develop.	Exp.	pSBR	pSBR-CT$^{\Delta A1}$
SBR	SBR-MAb	AgI/II-PAb	No. 1	1.68	1.18
			No. 2	1.33	1.23
			No. 3	1.51	1.07
			No. 4	1.13	1.14
			Mean ± SD	1.39 ± 0.21	1.16 ± 0.07
SBR-CT$^{\Delta A1}$	Gm1	AgI/II-PAb	No. 1	0**	2.03
			No. 2	0	3.05
			No. 3	0	3.49
			No. 4	0	2.13
			Mean ± SD	0	2.68 ± 0.71

[a]amount of recombinant protein in cell lysate of clones containing either pSBR or pSBR-CT$^{\Delta A1}$;
* = % SBR polypeptide/total soluble protein in cell lysates;
** = % SBR-CT$^{\Delta A1}$ chimeric protein/total soluble protein in cell lysates.

EXAMPLE 24

Serum IgG Antibody Responses

Oral immunization of mice with the *S. typhimurium* clones expressing SBR or SBR-CT$^{\Delta A1}$ resulted in dose-dependent primary serum IgG responses to AgI/II which were significantly enhanced after a single booster dose (FIG. 9A). Although the responses to AgI/II induced by i.g. administration of either *S. typhimurium* clone (i.e., expressing SBR alone or linked to CTA2/B) were not statistically different, a trend was observed towards higher serum IgG responses after two or three primary immunizations with *S. typhimurium* expressing SBR alone than with the clone expressing the SBR-CT$^{\Delta A1}$ chimeric protein (FIGS. 9B and 9C).

The response to the Salmonella vector was essentially similar with increasing number of doses (one to three) given during primary immunization (data shown for the groups which received two primary doses; FIG. 10A). As expected, serum IgG antibodies to CT were induced after immunization with the clone expressing the chimeric SBR-CT$^{\Delta A1}$ molecule but not with the clone expressing SBR alone (FIG. 10A). As with anti-AgI/II responses, the serum IgG response to CT was also significantly elevated following the booster immunization (FIG. 10A).

Analysis of serum samples obtained from i.n. immunized mice for anti-AgI/II responses, confirmed the trend of higher responses in mice immunized with *S. typhimurium* expressing SBR than SBR-CT$^{\Delta A1}$ seen in i.g. immunized animals (FIG. 11A). In this case the differences in anti-AgI/II responses between the clones were statistically significant except for the responses on week 17 (FIG. 11A). This trend did not seem to correlate with the anti-Salmonella responses induced by i.g or i.n. immunization. In fact, the anti-vector responses appeared to be higher for the SBR-CT$^{\Delta A1}$-producing clone; FIGS. 10A and 11B. The kinetics as well as the magnitude of the serum IgG responses to Salmonella and the cloned Ags after i.n. immunization (FIGS. 11A and 11B) were comparable to those after i.g. immunization (FIGS. 9A, 9B, 9C and 10A) despite that the number of Salmonellae given by the i.n. route was lower by one order of magnitude.

In the above experiments, preimmune serum samples (from 10- to 12-week old mice) did not contain detectable antibodies to AgI/II, CT, or Salmonella (corresponding data points at week 0 in FIGS. 9A, 9B, 9C, 10A, and 11A and 11B, represent the detection limit of the ELISA). Furthermore, 27 week-old unimmunized controls did not show substantial antibody levels to AgI/II or CT ($\leq$1.2 μg/ml) but they did develop a relatively weak response (compared to immunized animals) against Salmonella (17.8×/÷1.6 μg/ml, n=5), probably because of cross-reactions with related gram-negative bacteria of their normal flora.

In these studies antibody responses to the SBR of AgI/II were detected using native AgI/II as the coating agent in the ELISA, as recombinant vaccines should be able to induce responses against the antigen expressed by the pathogen. On a limited number of samples, the influence of coating with purified rSBR on the magnitude of the detected responses was also determined. ELISA with serum samples tested on plates coated with either rSBR or AgI/II showed that the antibody response to rSBR was about 2 times higher than to AgI/II (2.15×/÷1.49, n=13), implying substantial antigenic and possibly structural similarities between rSBR and the corresponding region (residues 186 to 577) of the whole AgI/II. Moreover, the antibody response against the CTA2/B component of the chimeric protein was about 3 times higher (3.34×/÷1.10, n=6) when detected with native CT bound to G_{M1} ganglioside-precoated plates than when CT was directly coated on microtiter plates.

EXAMPLE 25

IgG2a/IgG1 Profile for Vector and Carried Antigens

To determine the subclass distribution of serum IgG antibody responses, samples from mice orally immunized with SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* were analyzed for levels of IgG2a and IgG1 antibody responses which are indicative of a Th type-1 or Th type-2 response, respectively. Serum IgG antibodies to whole Salmonella belonged predominantly to the IgG2a subclass (IgG2a/IgG1>10) but a mixed IgG2a and IgG1 response pattern (IgG2a/IgG1=1) was observed for the cloned Ags, SBR and CTA2/B (TABLE V). Analysis of samples from a study in which mice were orally immunized with purified SBR-CT$^{\Delta A1}$, revealed predominant IgG1 responses to SBR and CTA2/B, indicating that immunization with the *S. typhimurium* vector shifted the responses towards the IgG2a subclass (TABLE V). In the case of the anti-SBR responses the shift was statistically significant. In terms of the IgG2a/IgG1 ratio, the response to SBR was not influenced by the route of immunization (i.g. or i.n.) or the presence or absence of CTA2/B (clones expressing SBR-CT/(^{A1} or SBR alone).

TABLE V

Profile of IgG2a/IgG1 antibody responses in serum after oral immunization with SBR-CT$^{\Delta A1}$ expressed in *S. typhimurium* or administered as purified immunogen

Immunization with	antibody response to:	IgG2a/IgG1*	No. of mice IgG2a/IgG>1
S. typhimurium vector (n = 16)	SBR	1.02 ± 2.26**	8/16
	BTA2/B	1.21 ± 2.6	10/16
	Salmonella	11.2 ± 2.21	16/16
Purified SBR-CT$^{\Delta A1}$ (n = 6)	SBR	0.3 ± 1.94	0/6
	CTA2/b	0.48 ± 1.73	1/6

* = ration obtained following quanfication of subclass-specific antibody levels
** = values are the geometric mean x/(SD of the IgG2a/IgG1 ratios of individual mice.

EXAMPLE 26

Salivary IgA Antibody Responses

Oral immunization of mice with recombinant Salmonella vector, one to three times, resulted in the induction of increasingly higher salivary IgA antibody responses to AgI/II (FIGS. 12A, 12B, and 12C). A single oral booster immunization resulted in augmented antibody levels in the groups immunized one or two times during priming, whereas the secondary response in the groups immunized three times for priming was slightly lower than the peak primary response at the time measured (FIG. 12C). All groups of mice displayed significantly higher antibody levels four weeks after the secondary immunization than immediately before the booster immunization. Interestingly, although the anti-AgI/II responses induced by the SBR- and the SBR-CT$^{\Delta A1}$-expressing clones were generally not significantly different (except for the responses of the groups immunized three times for primary immunization where differences reached statistical significance after the boosting (FIG. 12C)), they showed the opposite trend than that observed in the serum IgG responses, i.e., the presence of CTA2/B appeared to enhance the salivary IgA response to AgI/II. In contrast, the salivary IgA anti-vector responses induced by the two Salmonella clones were very similar (data shown for the groups which were given two primary doses; FIG. 10B). IgA antibodies to CT in saliva were detected after immunization with the SBR-CT$^{\Delta A1}$-expressing clone only, and were significantly elevated by secondary immunization (FIG. 10B). The salivary IgA responses to Salmonella and the cloned Ags after i.n. administration of the recombinant *S. typhimurium* (FIGS. 13A, 13B and 13C) displayed similar characteristics as after i.g. immunization and were comparable in magnitude despite the use of lower doses. The secondary response to CT and AgI/II reached significantly higher levels than the primary response, two and four weeks following the booster immunization, respectively. When saliva samples were normalized for total IgA content, samples from 27-week-old unimmunized mice showed similar levels of "background" antibody activity to the test Ags as the preimmune samples of immunized animals reported in FIGS. 10B, 12A, 12B, 12C, 13A, 13B, and 13C.

The finding that anti-AgI/II responses tended to be higher in saliva but lower in serum when mice were immunized with the SBR-CT$^{\Delta A1}$-expressing clone than with the clone producing SBR alone, was further supported by data from additional groups of mice. These mice were given a single immunization of 10^{10} CFU by the i.g. or 10^9 CFU by the i.n. route, boosted with the same dose 15 weeks later, and displayed the above mentioned trend regardless of the route of administration (FIGS. 14A and 14B). In the same experiment, the mice immunized with a single i.g. dose of 10^{10} CFU showed higher anti-AgI/II responses in serum and saliva than mice which received a single i.g. dose of 10^9 CFU (FIGS. 9A and 12A), but equal or slightly lower responses than mice given 3 i.g. doses of 10^9 CFU (FIGS. 9C and 12C).

EXAMPLE 27

Intestinal IgA Responses

IgA anti-AgI/II responses were also detected in fecal extracts from i.g. immunized mice (FIGS. 15A, 15B, and 15C). The kinetics of the responses induced by the two recombinant *S. typhimurium* clones also showed some trend for higher anti-AgI/II responses when the SBR antigen was co-expressed with CTA2/B (FIGS. 15A, 15B, and 15C). This trend was less pronounced than seen in saliva (FIGS. 12A, 12B, and 12C), and it did not show statistical significance except for two time points, i.e., FIG. 15A, at week 15 with groups given 1 primary dose, and, FIG. 15B, at week 19 with groups given 2 primary doses. At the same time, the anti-Salmonella response appeared to be higher in the case of the clone producing SBR alone (FIG. 10C), suggesting that the relatively high anti-AgI/II responses in the case of the SBR-CT$^{\Delta A1}$ clone may be related to the co-expression of SBR and CTA2/B. Intestinal IgA responses to Salmonella and the cloned Ags were also induced after immunization by the i.n. route (FIGS. 16A and 16B). As in the case of saliva, fecal samples from 27 week-old unimmunized mice showed similar levels of "background" antibody activity against the test Ags with the preimmune samples of immunized animals, but the background activity against CT appeared to be higher compared to that against AgI/II or even Salmonella (FIGS. 10C, 15A, 15B, 15C, 16A, and 16B).

Using a temperature-regulated expression system engineered in avirulent *S. typhimurium*, the present invention demonstrated high levels of antibodies against the cloned heterologous Ags in serum and mucosal secretions after oral or i.n. immunization. Expression of recombinant immunogens in this system was activated under in vivo conditions (37° C.), since at 37° C. target protein induction was shown to be optimal. This system was used to investigate whether the non-toxic A2/B moiety of cholera toxin can act as a Salmonella-cloned adjuvant when co-expressed with the SBR protein antigen. For this purpose, a *S. typhimurium* clone expressing SBR alone and a similar clone expressing the SBR-CT$^{\Delta A1}$ chimeric protein were used, which were found to produce similar levels of the SBR polypeptide. The amount of chimeric protein produced by the SBR-CT$^{\Delta A1}$

clone (2.68% of total soluble protein) was consistent with the estimated SBR levels (1.16%) expressed by the same clone, since SBR comprises 39% of the whole chimeric molecule by weight.

Quantitative analysis of serum samples showed that the IgG responses to SBR were generally lower after immunization with the SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* than with the clone expressing SBR alone. Although this may suggest intramolecular antigenic competition as observed within the IgG molecule, i.e., higher responses are induced to the Fab fragment when Fab is injected alone than when the whole IgG molecule is used for immunization, analysis of responses in secretions revealed an opposite trend. Comparing the mucosal IgA antibody levels to AgI/II induced by immunization with the two *S. typhimurium* clones at each time point examined (56 time-points including all groups from both i.g. and i.n. immunization; FIGS. 12A, 12B, 12C, 13B, 14B, 15A, 15B, 15C, 16A, and 16B), the response in the case of the clone expressing SBR linked to CTA2/B was higher on 51 occasions (91%). In contrast, the serum IgG response was higher in 83% of the occasions (31/36) in the case of the other clone, i.e., the one expressing SBR alone (FIGS. 9A, 9B, 9C, 11B, and 14A). One can speculate that CTA2/B may have a dual influence on anti-SBR responses arising from its dual role as an immunogen and as an immunoenhancing agent. As an immunogenic component of the SBR-CT$^{\Delta A1}$ chimeric molecule, CTA2/B may tend to depress the immune response to SBR through antigenic competition, and as a mucosal adjuvant it may tend to potentiate anti-SBR responses. This dual effect might have differentially influenced the observed mucosal and systemic responses if CTA2/B is able to provide better help for antibody production in mucosal inductive sites than in systemic compartments. Presumably, Salmonella-expressed CTA2/B can be delivered to both mucosal and systemic inductive sites because of the ability of the vector to colonize mucosal lymphoid tissues and to disseminate to systemic tissues. Interestingly, in an immunization study with influenza virus administered mucosally or systemically in the absence or presence of CTB, the adjuvant effect of CTB on antiviral antibody responses was found to be more pronounced after i.n. than after subcutaneous or i.p. immunization. These findings cannot be attributed to quantitative differences (equal doses were given by all routes and in the case of i.n. immunization the amount actually absorbed may be even less than that injected for systemic administration) but rather to a CTB adjuvant effect which is possibly influenced by the particular microenvironment where CTB acts.

Th1 or Th2 cells induce antigen-specific B cells to selectively produce IgG2a or IgG1 antibodies, respectively. Salmonella (as well as other intracellular microorganisms) generally induces a Th1-type response characterized by high levels of IFN-γ and IgG2a antibodies. The serum IgG response to the *S. typhimurium* vector displayed a high (>10) IgG2a/IgG1 ratio. Moreover, a mixed IgG2a and IgG1 response (IgG2a/IgG1≈1) was induced against Salmonella-expressed SBR, although a predominant IgG1 response to SBR was observed after oral immunization with purified SBR-CT$^{\Delta A1}$. These data also suggest that the type of response to a Salmonella-delivered protein antigen was not entirely determined by the vector but is also influenced by inherent properties of the cloned antigen.

The mechanisms for inducing intestinal IgA responses to orally administered vaccines have been extensively studied. Although less is known regarding responses induced after i.n. immunization, several mechanisms can be offered for the observed intestinal IgA responses following i.n. administration of recombinant Salmonella in mice. Antigenic stimulation of the nasal-associated lymphoid tissues which show anatomical similarities with the Peyer's patches in the gut (e.g., lymphoid follicles covered by M cells) may result in the dissemination and homing of lymphoid cells to remote mucosal effector sites, including the intestinal lamina propria, in a fashion analogous to stimulation of the GALT. Moreover, a portion of the Salmonella inoculum may have been swallowed by the mice resulting in direct stimulation of the GALT. To minimize this possibility, a relatively small volume was slowly applied to the external nares (~10 μl per nostril). If these mice did swallow some Salmonella organisms, the number would be relatively small compared to the oral dose given to i.g. immunized mice, i.e., 10 times higher than the i.n. dose. Alternatively, *S. typhimurium* could access the GALT by dissemination from nasal lymphoid tissues. Immunization by the i.n. route was generally as effective as by the i.g. route, despite using lower doses.

Besides a remarkable secondary IgG response in serum against AgI/II and CT, a pronounced secondary salivary IgA response was induced against the cloned Ags after i.n. and i.g. (especially when two primary doses were given) immunization, suggesting induction of immunological memory. Enhanced salivary IgA anamnestic responses to SBR or CTA2/B were not observed previously in mucosal immunization experiments using purified SBR-CT$^{\Delta A1}$ or the whole AgI/II molecule chemically conjugated to native CTB. Because of concerns regarding the efficacy of repeated use of Salmonella as a carrier for various heterologous Ags, it was of interest that boosting of mucosal IgA and serum IgG antibody responses was induced after i.g. booster immunization of mice with a pre-existing intestinal IgA response to the Salmonella vector.

In summary, despite the requirement for genetic coupling of CTA2/B to SBR to induce substantial anti-SBR responses after mucosal immunization with purified immunogen, expression of SBR alone in an avirulent *S. typhimurium* vector was sufficient to induce high levels of antibodies in serum and mucosal secretions. The finding that the immunogenicity of Salmonella-delivered SBR was not significantly dependent on co-expression of CTA2/B, suggests that in oral immunization with purified SBR-CT$^{\Delta A1}$ targeting of SBR to the GALT via G_{M1} receptors on the overlying antigen-sampling M cells, may constitute an important immunoenhancing mechanism. The requirement for this mechanism, which would also reduce the exposure of SBR to proteases in the gut lumen, is bypassed by the Salmonella vector because of its tropism for the GALT, where SBR will eventually be delivered. The current system can be modified so that CTA2/B can find application as a Salmonella-cloned adjuvant, especially for Ags that are poor immunogens when delivered by this live antigen-delivery system.

EXAMPLE 28

Intranasal Immunization of Rats with AgI/II, AgII, and SBR Chemical/Genetic Conjugates

Fischer rats, 19 days old, were used for intranasal immunization studies performed according to NIH guidelines and protocols approved by the UAB Institutional Animal Care and Use Committee. The animals were immunized 3 times at 14-day intervals with 50 μg of the appropriate inunuogen (see group designations), with or without an adjuvant amount (1 μg) of cholera toxin (CT), in a volume of 50 μl which was slowly applied in the external nares by means of a micropipettor.

For sampling and quantification of antibody responses, serum was obtained by centrifugation of blood samples

collected from the retroorbital plexus with heparinized capillary pipettes. Saliva samples were collected by means of a Pasteur pipette after stimulation of ovary flow by intraperitoneal injection of 10 μg carbachol. Samples were obtained 1 day before the immunizations and 2 weeks after the last immunization. The levels of isotype-specific antibodies from serum and saliva, and total salivary IgA were determined by ELISA on microtiter plates coated with native AGI/II, recombinant SBR, AgII, G_{MI} followed by CT, or goat anti-rat IgA. The plates were developed with the appropriate peroxidase-conjugated goat anti-rat immunoglobulin isotype (IgG for serum samples and IgA for saliva samples) and o-phenylenediamine substrate with H_2O_2. The concentration of antibodies/total immunoglobulin in test samples was calculated by interpolation on standard curves generated using a rat immunoglobulin reference serum and constructed by a computer program based on four parameter logistic algorithms.

The results that follow are presented as μg/ml of specific serum IgG antibody or as % specific salivary IgA antibody/total IgA. Antibody data were logarithmically transformed to normalize their distribution and homogenize the variances. The data were finally retransformed and presented as geometric means ×/+ standard deviation for ease of interpretation. The presented data are from samples obtained 2 weeks after the last immununization. Specific antibodies in preimmunune samples were not detectable.

Immunization groups 1, 2, and 3 are controls, groups 4 and 5 were immunized with SBR-CTA2/B chimeric protein (SBR-CT$^{\Delta A1}$, shown as CHIM) without (group 4) or with (group 5) CT as adjuvant, and groups 6–11 were immunized with chemical conjugates of SBR, AgII, or AgI/II coupled to recombinant CTB, without or with CT as an adjuvant.

Antibody responses were assayed against the intact AgI/II (FIGS. 17 and 21), as well as against SBR (FIGS. 18 and 22), and AGII (another part of AGJM distinct from the part containing SBR; FIGS. 19 and 23). Responses against CT (FIGS. 20 and 24) are given for comparison, since the immunogens as well as the adjuvant (CT) where used also induce responses to CT. Since CT is regarded as the most potent mucosal immunogen, these comparisons serve to put the magnitude of responses to SBR or Ag I in perspective.

In all instances, responses (measured against SBR, AgI/II, or AGII) to the various immunogens given without CT adjuvant were undetecable; the use of CT as adjuvant was necessary to obtain responses in these rats (Fischer strain). This finding is in marked contrast to all previous results obtained in mice (BALB/c strain), in which antibody responses to SBR-CTA2/B chimeric protein or to AgI/II conjugated chemically to rCTB given intranasally were generated in the absence of CT adjuvant. The reason for this difference of response is not clear, and it is not known if this reflects the particular strain of rat used, or is typical of rats as compared to mice. Reports of studies performed on human subjects indicate that humans respond well to intranasal or oral immunization with recombinant CTB, i.e., more like mice than rats. The following is a comparison of responses to the different immunogens for animals immunized with CT as adjuvant.

Both SBR (in the form of either the chimeric immunogen, group 5; or the chemical conjugate, group 7) and AGII (group 9) as well as the intact AgI/II (group 9) induced antibodies detectable against AgI/II in serum (IgG, FIG. 17) and in saliva (IgA, FIG., 21). The intact AgI/II and AgII (serum only) tended to induce the strongest responses measured in this way. However, SBR (in either form) induced the strongest responses measurable against SBR (FIGS. 18 and 22) whereas AgII failed to induce responses to SBR. Conversely, AgII induced responses measurable against AgII (FIGS. 19 and 23) but SBR did not. Despite the fact that the use of CT as an adjuvant also induced very strong responses to itself (FIGS. 20 and 24), the responses to SBR, AgI/II, and AgII compare well with the responses to CT.

Therefore, the chimeric immunogen, SBR-CTA2/B, is very effective for inducing serum IgG and salivary IgA antibodies to SBR, which also react with the parent antigen, AgI/II. This may be advantageous, because SBR was selected as a part of the, AgI/II molecule that appears to be functionally important for the adherence of *Streptococcus mutans* to tooth surfaces. Immunization with AgI/II appears to induce antibodies most strongly against the AgII part of the molecule, yet earlier work indicated that antibodies to AgII might not be protective against *S. mutans*-induced dental caries. The advantage of using genetically constructed chimeric immunogens may therefore include the ability to direct an antibody response to a functionally important part of tbe antigen molecule that is otherwise less immunogenic than parts of the the molecule that may be functionally less important. Such a finding may be of considerable importance in vaccine development, since surface molecules of microorganisms that have functional activity in pathogenesis may have evolved structures that divert host immune responses away from the more sensitive parts of the molecule.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

What is claimed is:

1. A method of producing an immune response by administration of an attenuated strain of bacteria, wherein said attenuated bacteria express an antigen of interest as a fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter; and,

c) DNA sequences encoding the antigen of interest, wherein said DNA sequences are fused in frame to the A2 subunit of cholera toxin.

2. The method of claim 1, wherein salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II) is the antigen of interest fused to the A2 subunit of cholera toxin.

3. The method of claim 1, wherein said plasmid further comprises DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein to facilitate assembly of a chimeric protein.

4. The method of claim 3, wherein said plasmid is pCT$^{\Delta A1}$.

5. The method of claim 3, wherein salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II) is the antigen of interest fused to the A2 subunit of cholera toxin.

6. The method of claim 5, wherein said plasmid is pSBR-CT^{AA1} (alternatively designated pSBR-CTA2/B).

7. The method of claim 3, wherein said attenuated bacterial strain is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, and subcutaneously.

8. The method of claim 3, wherein said immune response results in the production of antibodies to the protein antigen sequence in a bodily fluid selected from the group consisting of saliva, intestinal secretions, respiratory secretions, genital secretions, tears, milk and blood.

9. The method of claim 3, wherein said immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues of said individual, the development of cytotoxic T cells and immunological tolerance to the protein antigen sequence.

10. An attenuated Salmonella strain, wherein said Salmonella strain expresses a chimeric fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter;

c) DNA sequences encoding a fusion protein of the antigen of interest fused in frame with the A2 subunit of cholera toxin; and,

d) DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein of the antigen of interest and A2 cholera toxin subunit to facilitate assembly of a chimeric protein.

11. An attenuated Salmonella strain, wherein said Salmonella strain expresses a chimeric fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter;

c) DNA sequences encoding a fusion protein of salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused in frame to the A2 subunit of cholera toxin; and,

d) DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein of saliva binding protein and A2 cholera toxin subunit to facilitate assembly of a chimeric protein.

* * * * *

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Russell & Connell	§	ART UNIT: 1632
	§	
FILED: April 3 2001	§	EXAMINER:
	§	Li, Qian
SERIAL NO.: 09/825,105	§	
	§	
FOR: Chimeric Antigen-Enterotoxin Mucosal Immunogens	§	DOCKET: D6321
	§	

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ATTENTION: Board of Patent Appeals and Interferences

APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on October 7, 2003. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is the UAB Research Foundation.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Originally claims 1-29 were filed with this Application. Claims 4-5 and 7-23 were canceled by amendment. The pending claims 1-3, 6, 24-29 are being appealed of which claims 1, 24 and 27 are an independent claims.

IV. STATUS OF AMENDMENTS

Subsequent to the final rejection mailed May 6, 2003, Applicants submitted a Response After Final which canceled claims 9-23. All pending claims are shown in Appendix A.

V. SUMMARY OF THE INVENTION

The present invention provides methods of inducing immune responses by recombinant antigen-enterotoxin chimeric mucosal immunogens comprising the A2/B subunits of heat-labile type II toxins (see Abstract). The enzymatically active A1 subunit of heat-labile type II toxin was replaced with an immunogen such as the saliva-binding region (SBR) from the streptococcal adhesin AgI/II (page 6, lines 7-15). Intranasal immunization of BALB/c mice with the chimeric proteins induced significantly higher plasma and mucosal anti-SBR IgA and IgG antibody responses (page 40, lines 3-9).

The present invention indicates that heat-labile type II toxins (LT-IIa and LT-IIb) and cholera toxin (a type I heat-labile

enterotoxin) induced secretion of different cytokines from anti-CD3-stimulated human peripheral blood mononuclear cell cultures (page 54, lines 4-18), possibly due to differential effects on CD40L expression and IL-12 secretion (page 48, line 17 to page 49, line 5). Accordingly, chimeric immunogens comprising the A2/B subunits of heat-labile type II toxins may possess unique immunomodulatory properties on $CD4^+$ T cells due to secretion of Th1 cytokine (page 40, lines 19-21).

The present invention also indicates an advantage for chimeric immunogens based on heat-labile type II toxins in that these chimeric proteins induced substantially lower antibody responses to the enterotoxin components of the immunogens. This feature may permit repeated administration of heat-labile type II toxin-based chimeric immunogens without the loss of immunogenicity due to pre-existing antibodies against the enterotoxin (page 41, lines 1-6).

VI. ISSUES

35 U.S.C. §103

Whether claims 1-3, 6 and 24-29 are obvious over **Toida** et al. (*Infect. Immunity*, 1997) in view of **Rappuoli** et al. (*Immunol. Today*, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 1989; *Vaccine*, 1990) and **Connell** et al. (*Immuol. Lett.*, 1998; *Infect. Immunity*, 1992) under 35 U.S.C. §103(a).

Whether claims 1-3, 6 and 24-29 are obvious over **Russell** et al. (U.S. patent no. 6,030,624) in view of **Rappuoli** et al. (*Immunol. Today*, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 1989; *Vaccine*, 1990) and **Connell** et al. (*Immuol. Lett.*, 1998; *Infect. Immunity*, 1992) under 35 U.S.C. §103(a).

VII. GROUPING OF CLAIMS

The rejected claims do stand or fall together.

VIII. ARGUMENTS

Rejection Under 35 U.S.C. §103

In the Advisory Action mailed August 26, 2003, the Examiner maintained the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) as being unpatentable over **Toida** et al. (Infect. Immunity 65:909, 1997) in view of **Rappuoli** et al. (Immunol. Today 20:493, 1999), and further in view of **Schodel** et al. (Infect. Immunity, 57:1347, 1989; Vaccine 8:569, 1990) and **Connell** et al. (Immuol. Lett. 62:117, 1998; Infect. Immunity 60:1653, 1992). Applicant respectfully requests that this rejection be reversed.

The present invention is drawn to methods of inducing cellular immune response or Th1 immune response (i.e., T cell-mediated immunity) by a fusion protein comprising an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin.

In contrast, **Toida** et al. describe a method of inducing humoral (antibody) and cellular (T helper cell) immune responses using a chimeric immunogen comprising an antigen fused to the A2 and B subunits of cholera toxin (a type I heat-labile enterotoxin). **Rappuoli** et al. describe the the structure and mucosal

adjuvanticity of cholera and *E. coli* heat labile enterotoxin. **Schodel** et al. describe inducing T cell immune responses (but not humoral immune response) against an antigen using a fusion protein which consists of an antigen fused to the B subunit of *E. coli* heat labile enterotoxin.

Connell et al. (1992) describe the structural characterization of hybrid toxins produced by assembly of A and B polypeptides from type I and type II heat labile enterotoxins. **Connell** et al. (1998) describe the induction of humoral immune response upon co-administration of a weak immunogen and a type II heat labile enterotoxin.

Rappuoli et al. and **Schodel** et al. only teach heat labile enterotoxins in general without discussing any similarities and differences between type I and type II heat labile enterotoxins. The Examiner acknowledges that **Rappuoli** et al. and **Schodel** et al. do not distinguish type II from type I heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4). The Examiner argues, however, the gap in teaching on type II heat-labile enterotoxin can be filled by **Connell** et al. The Examiner's rejection is based on the assertion that **Connell** et al. compensate for the lack of teaching on type II heat labile enterotoxins in **Rappuoli** et al. and **Schodel** et al.

Accordingly, Applicant focuses the following discussion on **Connell** et al.

Asserting that **Connell** et al. (1992) teach structural compatibility between type I and type II heat labile enterotoxins, and **Connell** et al. (1998) teach type I and type II heat labile enterotoxins can be used as mucosal adjuvants, the Examiner concludes that apparently there is not much differences in adjuvant properties between type I and type II heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4). Hence, according to the Examiner, it would have been obvious to replace the type I heat labile enterotoxin in **Toida** et al. with a type II heat labile enterotoxins. Applicant submits that the Examiner's broad assertion of same adjuvant properties between type I and type II heat labile enterotoxins is not supported by the cited references and lacks a scientific basis.

There are significant structural differences between type I and type II heat labile enterotoxins. Within the A and B polypeptides of the enterotoxins, only the A1 fragments are homologous between type I and type II heat labile enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 13-20). The A2 fragments are much less homologous than the A1 fragments, and

the B polypeptides of type I enterotoxins have little or no significant homology with those of type II enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 20-24).

The Examiner acknowledges there is significant structural differences between type I and type II heat labile enterotoxins (Final Office Action mailed May 6, 2003, page 4; Advisory Action mailed August 26, 2003). The Examiner asserts, however, the **Connell** et al. references were cited to show type I and type II heat labile enterotoxins possess similar biological activities in spite of the structural differences (Advisory Action mailed August 26, 2003, citing first three rows of Table 2 in **Connell** et al., 1992).

Applicant submits that the term "similar biological activities" needs to be clarified in terms of what were taught in **Connell** et al. **Connell** et al. (1992) disclosed biological activities in terms of toxicity and structural integrity which was determined via recognition by enterotoxin subunit-specific antibodies (Table 2, **Connell** et al., 1992). **Connell** et al. (1998) taught induction of humoral immune response. Hence, in view of the significant structural differences between type I and type II heat labile enterotoxins, **Connell** et al. (1992, 1998) teach similar properties in toxicity, structural integrity and the ability to induce humoral

immune response. However, biological functions on toxicity, structural integrity and induction of humoral immune response do not provide any scientific basis for predicting the likelihood of inducing cellular or T cell-mediated immune response by type II heat labile enterotoxins. **Connell** et al. or other references cited by the Examiner do not provide any teaching or guidance on how to relate the properties of toxicity, structural integrity and induction of humoral immune response to the capacity of inducing cellular immune response. In other words, even though type I and type II heat labile enterotoxins have similar properties in toxicity, structural integrity and induction of humoral immune response, that does not mean these two types of enterotoxins would have similar properties in the induction of cellular immune response because the recited similar activities do not have direct and logical relationship to the capacity of inducing cellular immune response.

A person having ordinary skill in this art would readily recognize that toxicity and immunity are two distinct and separate areas of biological activities. Regarding the teaching on induction of humoral immunity, it is important to recognize the distinct features of humoral immunity (antibody immune response) vs. cellular immunity (T cell-mediated immune responses). The distinction

between humoral immunity and cellular immunity is significant and important. It is a basic concept in immunology that these two kinds of immune responses are stimulated by different immunogenic peptides (Class I MHC-restricted peptides vs. Class II MHC-restricted peptides) and are mediated by different immune effector cells (T cells vs. B cells). Hence, the parameters for the induction of humoral immunity are different from that for the induction of cellular immunity. One of ordinary skill in the art would readily recognize that parameters for the induction of humoral immunity are mostly, if not all, not suitable or applicable for the induction of cellular immunity. Therefore, the teaching on the induction of humoral immunity as disclosed in **Connell** et al. (1998) does not shed any light on whether an immunogen comprising a type II heat labile enterotoxin can induce cellular immune responses. Accordingly, the issue of whether an immunogen (in the present case, a type II heat labile enterotoxin) can induce an immune response (in the present case, cellular immunity) has to be resolved by actual experimentation.

The need for empirical experiments is also highlighted by the significant structural differences between type I and type II heat labile enterotoxins. The present invention relates to induction of

cellular immune response by a chimeric antigen comprising the A2 and B subunits of type II heat-labile enterotoxin. In contrast, the cited prior art references teach induction of cellular immune response by a chimeric antigen comprising the A2 and B subunits of type I heat-labile enterotoxin (**Toida** et al.). As discussed above, the A2 and B subunits of type I heat-labile enterotoxin have little or no significant homology with those of type II enterotoxins (**Connell** et al., 1992, page 1653, right column, lines 20-24). The cited prior art references do not provide any logical or scientific reasoning that would have indicated to a person having ordinary skill in this art that the A2 and B subunits of type II heat-labile enterotoxin would induce cellular immune response in a way similar to that induced by the A2 and B subunits of type I heat-labile enterotoxin.

In conclusion, the combined prior art references teach that the A2 and B subunits of type I heat labile enterotoxin are structurally different from that of type II heat labile enterotoxin, and these two types of enterotoxins possess similar activities in terms of toxicity, structural integrity and induction of humoral immune response (**Toida** et al., **Connell** et al., 1992, 1998). The lack of differentiation between type I and type II heat labile enterotoxins in **Rappuoli** et al. and **Schodel** et al. renders these

two references ambiguous and useless in providing any guidance. The combined prior art references, however, do not teach or suggest that the A2 and B subunits of type I and II heat-labile enterotoxins would have similar capacity in the induction of cellular immune response in spite of substantial structural differences. Hence, the cited prior art references do not provide one of ordinary skill in the art with the requisite reasonable expectation of successfully producing Applicant's claimed methods. Accordingly, the invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

The Examiner also contends that the instant situation is amenable to the type of analysis set forth in In re Kerkhoven, 205 USPQ 1069 (CCPA 1980) wherein the court held that it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose. Applicant respectfully disagrees. Applicant submits that the present invention is not amenable to the type of analysis set forth in In re Kerkhoven because, as discussed above, the prior art references do not teach or suggest two compositions (the A2 and B subunits of type I heat-labile enterotoxin vs. that of type II enterotoxin) each of which is useful for the same purpose (i.e. induction of cellular immune

response). Accordingly, Applicant submits that the invention as a whole is not *prima facie* obvious, and that the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) be reversed.

In the Advisory Action mailed August 26, 2003, the Examiner maintained the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) as being unpatentable over **Russell** et al. (US patent no. 6,030,624) in view of **Rappuoli** et al. (*Immunol. Today* 20:493, 1999), and further in view of **Schodel** et al. (*Infect. Immunity*, 57:1347, 1989; *Vaccine* 8:569, 1990) and **Connell** et al. (*Immuol. Lett.* 62:117, 1998; *Infect. Immunity* 60:1653, 1992). Applicant respectfully requestes that the Board reverse this rejection.

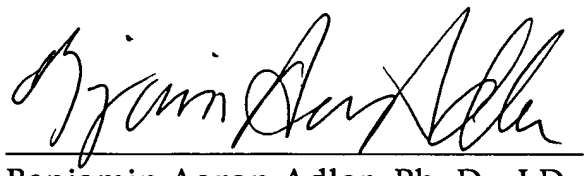
Russell et al. describe a method of inducing humoral (antibody) and cellular (T cell) immune responses using a chimeric immunogen comprising an antigen fused to the A2 and B subunits of cholera toxin (a type I heat-labile enterotoxin). The other cited references have been discussed above.

The Examiner rejects claims 1-3, 6 and 24-29 on the same basis as that based on **Toida** et al., **Rappuoli** et al., **Schodel** et al. and **Connell** et al. Therefore, the above discussion applies

here also. Applicant reiterates that the cited prior art references do not teach or suggest that the A2 and B subunits of type I and II heat-labile enterotoxins have similar capacity to induce cellular immune response in spite of substantial structural differences. The issue of whether the A2 and B subunits of type II heat labile enterotoxin can induce cellular immunity has to be determined by actual experimentation. The invention as a whole is not *prima facie* obvious to one of ordinary skill in the art at the time the invention was made. Accordingly, Applicant respectfully requests that the rejection of claims 1-3, 6 and 24-29 under 35 U.S.C. §103(a) be reversed.

Respectfully submitted,

Date: Dec 5, 2003



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CLAIMS ON APPEAL

1. A method of inducing an immune response by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin, wherein said immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues, the development of cytotoxic T cells and immunological tolerance to the antigen sequence.

2. The method of claim 1, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

3. The method of claim 1, wherein said type II heat-labile enterotoxin is selected from the group consisting of *E. coli* heat-labile type IIa toxin and *E. coli* heat-labile type IIb toxin.

6. The method of claim 1, wherein said immunogen is administered by a route selected from the group consisting of

orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

24. A method of increasing Th1 response and cell-mediated immunity by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a type II heat-labile enterotoxin.

25. The method of claim 24, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

26. The method of claim 24, wherein said immunogen is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

27. A method of increasing Th1 response and cell-mediated immunity by administration of a recombinant immunogen comprising a fusion protein of an antigen fused to the A2 and B subunits of a *E. coli* heat-labile type IIa or type IIb toxin.

28. The method of claim 27, wherein said antigen of interest is salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II).

29. The method of claim 27, wherein said immunogen is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, transcutaneously and subcutaneously.

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Oral Immunization with the Saliva-Binding Region of *Streptococcus mutans* AgI/II Genetically Coupled to the Cholera Toxin B Subunit Elicits T-Helper-Cell Responses in Gut-Associated Lymphoid Tissues

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Mice immunized intragastrically (i.g.) with a genetically constructed chimeric protein consisting of the saliva-binding region (SBR) of *Streptococcus mutans* AgI/II coupled to cholera toxin (CT) A2 and B subunits (CTA2/B) develop serum immunoglobulin G (IgG) and mucosal IgA antibody responses against AgI/II that are enhanced by the coadministration of CT as an adjuvant. To investigate the development of antigen-specific T cells in the gut-associated lymphoid tissues, mice were immunized i.g. with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT. AgI/II-specific T cells in Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen were assayed by lymphoproliferation and flow cytometry for the expression of T-cell surface markers, and cytokine mRNA expression was evaluated by reverse transcription-PCR. T-cell responses were consistent with antibody responses but were detectable after the first immunization. Proliferative responses of PP and MLN cells upon stimulation with AgI/II in vitro were low and delayed in mice given SBR alone, and these cells displayed a mixed type 1 and 2 (or Th0) pattern of cytokine expression. Immunization with SBR-CTA2/B resulted in greater AgI/II-specific proliferative responses in PP cells and an increase in the proportion of $CD4^+$ T cells. Coadministration of CT with SBR-CTA2/B led to greater proliferative responses especially in the MLN cells, which then showed an increase in $CD4^+$ cells. Immunization with SBR-CTA2/B (with or without CT) skewed the cytokine expression pattern in PP and MLN cells toward Th2. The results indicate that T helper cells were induced in gut-associated lymphoid tissues by i.g. immunization with SBR-CTA2/B, concomitantly with and prior to the appearance of circulating and mucosal antibodies.

Initial adherence of *Streptococcus mutans* to tooth surfaces appears to be mediated largely by the 167-kDa surface fibrillar adhesin known as AgI/II (synonyms, antigen B, P1, SpaP, and PAc) (14). The adhesion domain that interacts with salivary pellicle has been located to the alanine-rich (A) repeat region in the N-terminal part of the molecule (2, 12) extending from the cell surface probably in an α-helical conformation (21). Early studies on AgI/II indicated that rhesus monkeys immunized with *S. mutans* and showing protection against dental caries mounted antibody responses especially against the complete molecule rather than against AgII (34), which corresponds to the C-terminal one-third. These results were supported by the finding that immunization with either complete AgI/II or the isolated AgI component (corresponding to the N-terminal two-thirds) afforded protection against caries (22). Thus, a rational approach to immunization against *S. mutans*-induced dental caries can be based on the generation of an appropriate antibody response in the saliva that would inhibit the adherence of *S. mutans* to tooth surfaces. Human secretory immunoglobulin A (S-IgA) antibodies to AgI/II have been shown to inhibit such adherence (14). However, S-IgA antibodies in saliva and other secretions are not effectively induced by conventional parenteral immunization (27).

S-IgA antibodies are most effectively induced by stimulating the common mucosal immune system (27), for example, by enteric immunization which stimulates the gut-associated lymphoid tissues, including the Peyer's patches (PP) of the small intestine. Considerable attention has been given to the development of improved procedures for the oral delivery of vaccines (28), one of which is coupling antigens to the nontoxic binding B subunit of cholera toxin (CT), a safe and highly immunogenic protein in humans (16). The B subunit of CT (CTB), because of its avid binding to G_{M1} ganglioside, which is present on all nucleated cell surfaces, is readily taken up by the M cells covering PP and passed to the underlying immunocompetent cells which initiate the mucosal IgA antibody response. Antigen-stimulated IgA-committed B cells, and corresponding T helper cells, then emigrate via draining lymphatics to the mesenteric lymph nodes (MLN) and thence via the thoracic duct to the circulation before relocating in the effector sites of mucosal immunity, such as the salivary glands. Here terminal differentiation of B cells into IgA-secreting plasma cells occurs, and their product, polymeric IgA, is transported through the glandular epithelium to form S-IgA. Several studies have shown that other antigens can be coupled to CTB to generate strong mucosal IgA antibody responses to the desired antigen (4, 26, 36) and that intact CT, though toxic, serves as an adjuvant that enhances the response to coadministered antigens (9, 23).

For immunization against *S. mutans*-induced caries, this laboratory has developed a potent oral immunogen consisting initially of AgI/II chemically coupled to CTB (20, 36, 43). Mice and monkeys immunized intragastrically (i.g.) or intranasally with this develop salivary IgA antibodies to AgI/II (35, 36, 43). Recently, a novel class of genetically engineered mucosal im-

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munogen, in which the 42-kDa saliva-binding region (SBR) of AgI/II is inserted in CT in place of the toxic CT A1 subunit to create a chimeric protein in which SBR is coupled to pentameric CTB by the CTA2 peptide, has been developed (11). This protein has been shown to induce a persistent salivary IgA antibody response against AgI/II when administered i.g.; CT coadministered as an adjuvant enhances the response. However, optimal responses to either the chemically conjugated AgI/II-CTB or the genetically constructed SBR-CTA2/B have appeared to require three doses given at 10-day intervals (11, 36). This finding implies that the first dose must prime the mucosal immune system to respond to second and subsequent doses of the immunogen. Such priming would be expected to elicit antigen-specific T helper cells in the PP and MLN. Furthermore, the enhancement of antibody responses by the adjuvant effect of CT might be expected to involve increased T-helper-cell activity. This study was undertaken to test these hypotheses by examining AgI/II-responsive T cells in the PP and MLN of mice during the course of i.g. immunization with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant.

MATERIALS AND METHODS

Antigens. AgI/II was purified chromatographically from the culture supernatant of *S. mutans* essentially as described previously (33).

The SBR-CTA2/B chimeric protein was constructed and expressed in *Escherichia coli* and purified from extracts as described previously (11). In essence, this procedure consisted of PCR amplifying DNA for a 42-kDa segment encompassing the A repeat region and some downstream sequence of AgI/II from the *pac* gene, ligating this in a modified pET20b(+) plasmid (Novagen, Inc., Madison, Wis.) in frame with and upstream of the genes for CTA2 and CTB, and transforming the recombinant plasmid into *E. coli* BL21(DE3) cells (Novagen).

SBR polypeptide was obtained by excising the relevant DNA and religating it into unmodified pET20b(+) in order to express SBR with a six-residue histidine sequence derived from the plasmid. This plasmid was also expressed in *E. coli* BL21(DE3), and SBR was purified from cell lysates by metal chelation chromatography on a nickel-loaded column (Novagen) according to the manufacturer's instructions.

CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell, Calif.).

Animals and immunization. Adult BALB/c mice of either sex, 14 to 20 weeks old, from a pathogen-free colony were used for all experiments. Groups of nine mice were immunized i.g. three times at 10-day intervals by gastric intubation of either SBR-CTA2/B (100 μg) alone, SBR-CTA2/B together with 5 μg of CT as an adjuvant, or an equimolar amount of SBR (40 μg), all given in 0.5 ml of 0.35 M $NaHCO_3$. Serum and saliva samples were collected on day 0 and 10 days after each immunization for assay of antibodies by enzyme-linked immunosorbent assay (ELISA). In some experiments, subgroups of three mice were killed 10 days after each immunization for the preparation of cells from PP, MLN, and spleens for T-cell proliferation and flow cytometric analyses.

ELISA. Serum IgG and salivary IgA antibodies to AgI/II and total salivary IgA concentrations were determined by ELISA as described previously (36) on plates coated with AgI/II and anti-mouse IgA, respectively, and by using goat anti-mouse IgG- and IgA-peroxidase conjugates (Southern Biotechnology Associates, Inc., Birmingham, Ala.) as detection reagents. Unknowns were interpolated on calibration curves constructed by a computer program based on four-parameter logistic algorithms as previously described (36).

Preparation and culture of lymphoid cells. Single-cell suspensions were obtained by teasing PP, MLN, and spleen apart with needles, and tissue debris was removed by filtering through nylon mesh. Peripheral blood mononuclear cells were obtained by centrifugation on Histopaque 1083 (Sigma Diagnostic, St. Louis, Mo.). Remaining erythrocytes were lysed in buffered ammonium chloride; the cells were washed three times in RPMI 1640 medium (Mediatech, Washington, D.C.) supplemented with 2% fetal calf serum (FCS) and were finally resuspended in 10% FCS–RPMI 1640. Cells were cultured in 10% FCS–RPMI 1640 supplemented with 1 mM sodium pyruvate, nonessential amino acids, 2 mM glutamine, 100 U of penicillin-streptomycin per ml, 25 mM HEPES, and 0.01 mM 2-mercaptoethanol.

Flow cytometry. Cell marker expression on freshly isolated cells was determined by double staining with biotinylated anti-CD4 (GK1.5) followed by avidin-phycoerythrin and either fluorescein isothiocyanate-conjugated anti-CD3 (145-2C11) or fluorescein isothiocyanate-conjugated anti-CD8 (53-6.72) and by incubation for 30 min at 4°C in 2% FCS–Dulbecco's phosphate-buffered saline with 0.02% NaN_3. Cells were washed, fixed in 1% paraformaldehyde overnight, and analyzed on a FACStar IV flow cytometer (Becton Dickinson, Mountain View, Calif.).

Proliferation assay. Cells from PP, MLN, and spleens were incubated at 10^5 cells/well (0.1 ml) in triplicate with a previously optimized concentration of AgI/II (0.5 μg/ml) for 5 days and were pulsed with [^{3}H]thymidine (0.5 μCi/well) 8 h before harvesting. Uptake of ^{3}H was counted by a liquid scintillation counter. The stimulation index was calculated as cpm (wells with AgI/II)/mean cpm (control wells).

Cytokine expression. The expression of cytokines by PP, MLN, and spleen cells after culture in vitro with or without AgI/II (0.1 μg/ml) for 24 h was determined by a reverse transcription (RT)-PCR procedure for the amplification of cytokine mRNA. Cells (5×10^6 to 7×10^6) were harvested from the cultures, washed thoroughly, and then lysed in 350 μl of lysing buffer for isolation of RNA, using an RNeasy kit (Qiagen Inc., Chatsworth, Calif.). RNA was redissolved in 40 μl of diethyl pyrocarbonate-treated water, and 2-μl samples were added to 18 μl of RT mixture (Perkin-Elmer, Foster City, Calif.) containing 1× PCR buffer, 5 mM $MgCl_2$, 1 mM (each) deoxyribonucleoside triphosphate, 1 U of RNase inhibitor per ml, 2.5 U of Moloney murine leukemia virus reverse transcriptase per ml, and 2.5 mM oligo$(dT)_{16}$. Mixtures were overlaid with 50 μl of light mineral oil and incubated in a thermal cycler (Perkin-Elmer) for 15 min at 42°C, 45 min at 37°C, 5 min at 99°C, and 5 min at 4°C. After RT 80 μl of PCR mixture (Perkin-Elmer) was added to each tube to give final concentrations of 25 U of AmpliTaq DNA polymerase per ml, 0.15 μM 5′ primer, 0.15 μM 3′ primer, 2 mM $MgCl_2$, and 1× PCR buffer II. Primers specific for murine gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, and β-actin (30) were obtained from Clontech Laboratories Inc. (Palo Alto, Calif.) or the Oligonucleotide Synthesis Core Facility of the UAB Comprehensive Cancer Center, and their specificities were verified by means of RT-PCR on RNA extracted from mitogen-stimulated mouse spleen cells. After heating at 95°C for 2 min, cDNA was amplified for 35 cycles consisting of 45 s at 94°C, 3 min at 72°C, and 2 min at 60°C. The products of amplification were analyzed by 2% agarose gel electrophoresis, revealed by ethidium bromide staining, and photographed by UV transillumination. The results were scored according to the presence or absence of a band of appropriate molecular size.

Statistical methods. Quantitative results were evaluated by Student's *t* test, by means of MultiStat (Biosoft, Ferguson, Mo.) on a Macintosh computer. Antibody data were transformed logarithmically to normalize their distribution and homogenize the variances.

RESULTS

Antibody responses. Intragastric immunization of mice with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT incrementally induced serum IgG and salivary IgA antibodies measured against whole AgI/II (Fig. 1). Immunization with SBR alone resulted in weak but statistically significant ($P < 0.001$ at all intervals) serum IgG antibody responses and modest salivary IgA antibodies that were significantly elevated above the background level only after the second and third immunizations ($P < 0.001$ and $P < 0.01$, respectively). Administration of the SBR-CTA2/B chimeric protein generated significantly greater serum IgG responses ($P < 0.001$), and coadministration of CT as an adjuvant further enhanced both the level and the earlier development of serum IgG antibodies. Salivary IgA antibodies also tended to be elevated by immunization with SBR-CTA2/B chimeric protein, especially when given with CT as an adjuvant; however, because of variation between animals, statistical significance was attained only after two doses given with CT. Nevertheless, the general pattern of results was in accordance with expectations based on responses to AgI/II, either alone or chemically conjugated to CTB, administered i.g. without or with CT adjuvant (4, 36). Total salivary IgA concentrations also increased in all animals during the immunization period, from 2.13 ± 0.61 μg/ml in unimmunized animals to 5.92 ± 0.64 μg/ml after three immunizations, but there were no significant differences between the immunization groups.

T-cell proliferative responses. To test whether T cells capable of proliferating in vitro in response to stimulation with AgI/II had been induced by the first, second, or third i.g. dose, groups of three mice were killed 10 days after a first, second, or third immunization with each immunogen preparation. This time interval corresponded to the immunization interval and was chosen to permit assessment of the potential responsiveness of cells sensitized by the previous immunization to the next dose. Mononuclear cells from PP, MLN, and spleens were

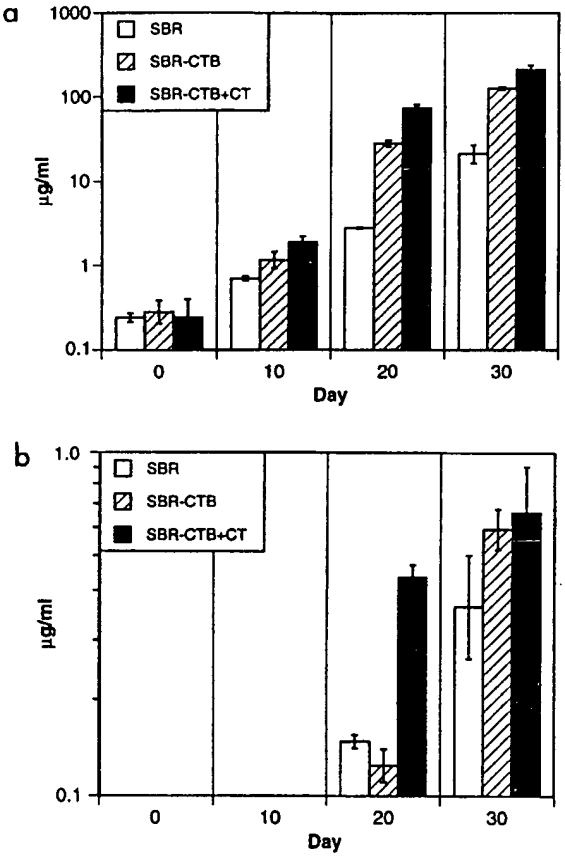

FIG. 1. Serum IgG (a) and salivary IgA (b) antibody responses to AgI/II in unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Immunizations were given on days 0, 10, and 20, and samples were collected 10 days after each immunization, i.e., on days 0 (unimmunized mice), 10 (one dose), 20 (2 doses), and 30 (3 doses). Results shown are the geometric mean and standard deviation, plotted on logarithmic ordinates, of samples from three animals analyzed separately. Salivary IgA antibodies were below detectable levels (<0.1 μg/ml) on days 0 and 10.

cultured with or without AgI/II. Incorporation of [^{3}H]thymidine expressed as stimulation indices revealed that AgI/II-responsive cells were elicited in the lymphoid tissues associated with the intestine, incrementally with the number and form of the immunogen doses (Fig. 2). PP and MLN cells taken from mice given two or three doses of SBR or of SBR-CTA2/B alone showed modest proliferative responses to AgI/II in vitro (stimulation indices in the range of 2.4 to 3.2; 5.44 for PP from mice given three doses of SBR-CTA2/B), whereas PP and MLN cells from mice immunized with SBR-CTA2/B plus CT adjuvant showed proliferative responses after one dose (stimulation indices of 2.3 and 3.6, respectively) and greater responses after two or three doses (stimulation indices of 3.1 to 6.1). The proliferative responses of PP and MLN cells were different: MLN cells responded similarly to (or less than) PP cells when taken from mice immunized with SBR or SBR-CTA2/B but showed greater responses to AgI/II in vitro when taken from mice given AgI/II-CTA2/B plus CT. Spleen cells generally did not respond to stimulation with AgI/II in vitro (stimulation indices of <2), except for those taken from mice immunized once with SBR-CTA2/B plus CT (stimulation index of 2.8). Cells from the PP, MLN, or spleens of unimmunized mice did not proliferate in response to AgI/II in vitro (stimulation indices of 1.2 to 1.5).

T-cell surface marker analysis. To elucidate the nature of the T-cell responses to i.g. immunization, cells freshly isolated from PP, MLN, spleen, or peripheral blood of mice immunized once, twice, or three times with the different immunogens were analyzed by flow cytometry for the proportion of cells expressing T-cell marker CD3 (all T cells), CD4 (T helper phenotype), or CD8 (T suppressor/cytotoxic phenotype). The results are shown in Fig. 3. Among PP cells, there was an increase in the proportion of total T cells after each immunization that was most noticeable in animals immunized with SBR-CTA2/B or SBR-CTA2/B plus CT; this increase was mostly in the CD4$^+$ T helper population, whereas the CD8$^+$ T suppressor/cytotoxic population remained small. The MLN cell populations remained more stable, except in the case of cells from mice immunized with SBR-CTA2/B plus CT, in which the CD4$^+$ population increased with the number of immunizations. MLN generally, however, contained more T cells of both phenotypes than PP, regardless of immunization status. Peripheral blood cells tended to show the greatest increases in the proportion of CD4$^+$ T cells after immunization, especially with SBR-CTA2/B plus CT, although these numbers must be interpreted with caution because of the small numbers of cells obtained. Spleen cells showed modest increases in the proportions of CD4$^+$ T cells after immunization in all groups.

Cytokine expression. To elucidate the pattern of expression of cytokines, PP, MLN, and spleen cells were taken from mice immunized three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT 3 days after the last dose, cultured in vitro for 24 h with or without AgI/II, and examined for the presence of mRNAs for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and β-actin by RT-PCR. This time interval was chosen because previous experience indicated that ex vivo analysis of cytokine production

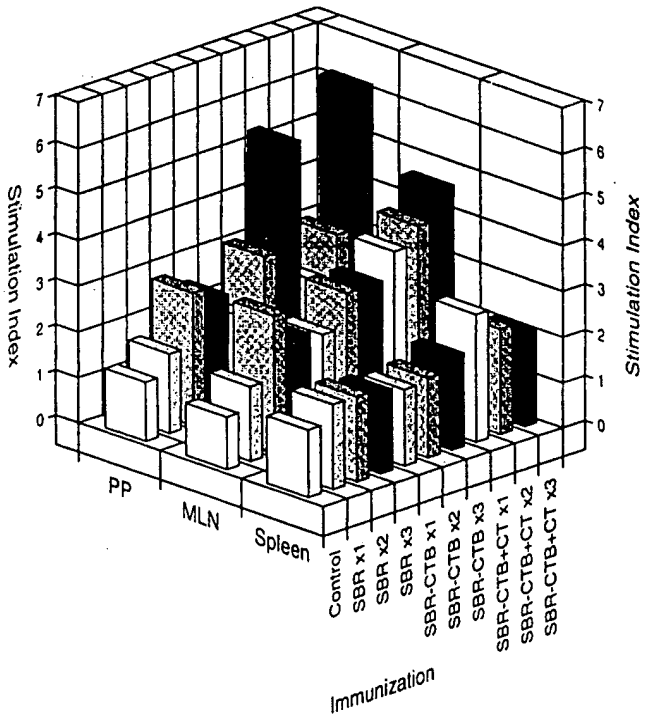

FIG. 2. Proliferative responses of cells from PP, MLN, and spleens of unimmunized (control) mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant, cultured in vitro with AgI/II. Results shown are mean stimulation indices of three replicate cultures; standard deviations ranged from ±0.04 to ±0.95.

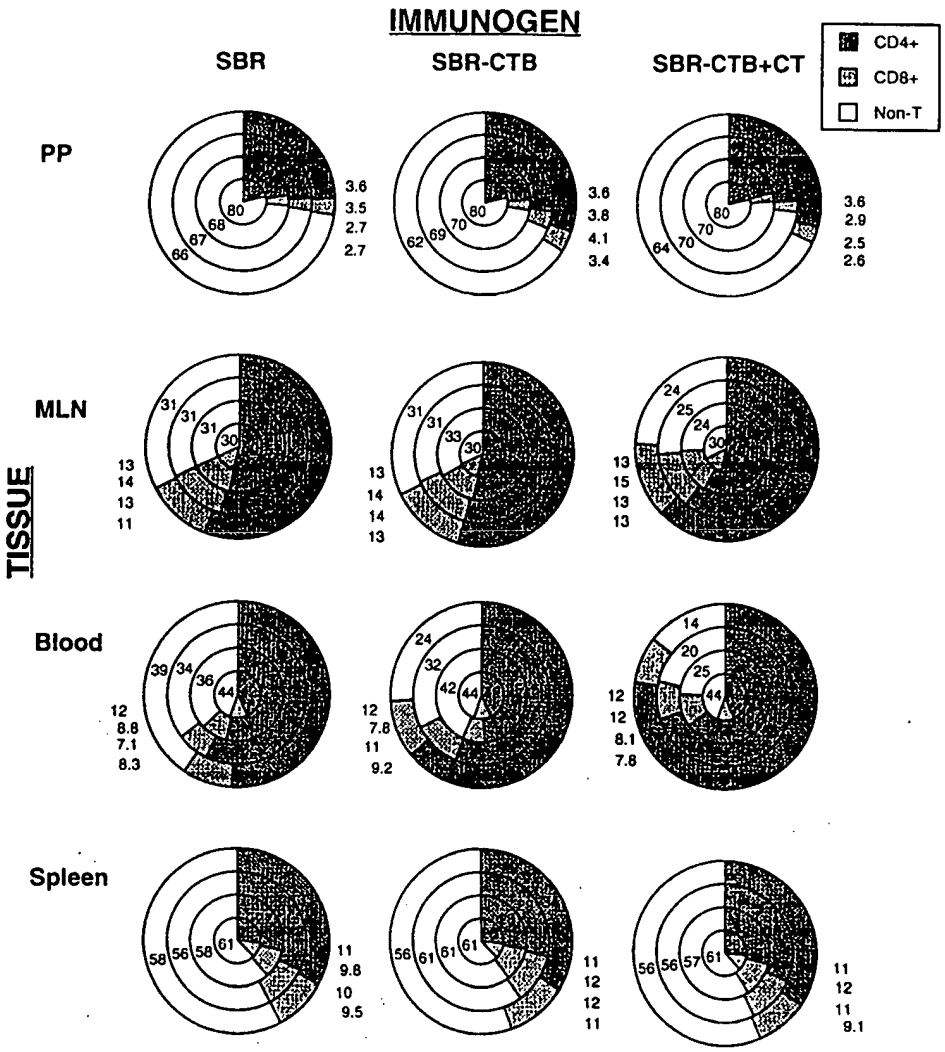

FIG. 3. Phenotypic analysis of cells from PP, MLN, peripheral blood, and spleens of unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Each pie shows the proportions of $CD4^+$, $CD8^+$, and $CD3^-$ (non-T) cells as a percentage of total gated mononuclear cells determined by flow cytometry, starting with unimmunized mice (center of each pie) and proceeding outward in concentric rings with mice immunized once, twice, or three times. Numbers within the rings are the individual percentages of each phenotype of cells (for clarity, $CD8^-$ cell data are shown outside the pies in descending order: zero, one, two, and three doses); the value shown for MLN from mice immunized once with SBR (marked as 51?) was not obtained experimentally but was inserted for plotting purposes as the average of the values either side of it. Note that the numbers in each ring of a pie do not sum to exactly 100% because of the presence of some $CD4^-$ $CD8^-$ (double-negative) $CD3^+$ T cells and possibly some $CD4^+$ $CD8^+$ (double-positive) T cells in each cell preparation.

was best assessed within a few days after immunization. All cultures generated β-actin PCR products of similar band intensities. After culture with AgI/II, PP, MLN, and spleen cells from mice immunized with SBR alone revealed mRNAs for IFN-γ and IL-2, but only PP and spleen cells also revealed IL-4 mRNA, whereas IL-5 mRNA was detectable in all cell cultures regardless of stimulation (Table 1). PP cells from mice immunized with SBR-CTA2/B, without or with CT adjuvant, did not reveal mRNA for IFN-γ or IL-2, even after culture with AgI/II, and MLN cells from these animals revealed variable IFN-γ and IL-2 mRNA responses. However, PP, MLN, and spleen cells revealed IL-4 mRNA particularly after stimulation with AgI/II, whereas all cultures were positive for IL-5 mRNA. Likewise, mRNAs for IL-6 and IL-10 were found in all cell cultures, regardless of immunization or in vitro stimulation (data not shown). Most notably, immunization with SBR-CTA2/B (without or with CT) resulted in a decrease of AgI/II-specific Th1 activity, as revealed by diminished expression of IFN-γ and IL-2 mRNAs in PP cells, and increased Th2 activity (IL-4 mRNA expression) in MLN cells in comparison with immunization with SBR alone (Table 1). There was an increase in IFN-γ and IL-2 expression (in response to stimulation with AgI/II in vitro) in PP, MLN, and spleen cells from mice immunized three times with SBR alone relative to cells from mice immunized twice (not shown). Likewise, spleen cells from mice immunized three times with SBR-CTA2/B (without or with CT) showed increased AgI/II-specific expression of IFN-γ, IL-2, and IL-4 relative to twice-immunized mice. Cells from unimmunized mice did not respond in culture with AgI/II by the expression of IFN-γ, IL-2, and IL-4 mRNAs above that revealed in control cultures, except that spleen cells showed weak evidence of IFN-γ expression upon culture with AgI/II. Thus, PP and MLN cells from mice immunized with SBR alone revealed type 1 (IFN-γ and IL-2) as well as type 2 (IL-4) cytokine responses upon stimulation in vitro, whereas cells from the same organs of mice immunized with SBR-CTA2/B

TABLE 1. Cytokine expression in PP, MLN, and spleen cell cultures of mice immunized with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT

Immunization[a]	Culture[b]	mRNA expression[c]											
		IFN-γ			IL-2			IL-4			IL-5		
		PP	MLN	Spleen	PP	MLN	Spleen	PP	MLN	Spleen	PP	MLN	Spleen
SBR	Control	−	−	−	−	−	−	−	−	−	+	+	+
	+AgI/II	+	+	+	+	+	+	+	−	+	+	+	+
SBR-CTA2/B	Control	−	−	−	−	−	−	+	−	+	+	+	+
	+AgI/II	−	−	+	−	+	+	+	+	+	+	+	+
SBR-CTA2/B + CT	Control	−	−	+	−	−	+	−	−	+	+	+	+
	+AgI/II	−	+	+	−	−	+	+	+	+	+	+	+

[a] Mice were immunized three times at 10-day intervals, and organs were collected 3 days after the last immunization.
[b] Cells were cultured in vitro for 24 h without (control) or with AgI/II (0.1 μg/ml).
[c] Cytokine mRNA expression detected by RT-PCR and scored according to the presence of an ethidium bromide-stained band of appropriate molecular size. −, no detectable band; +, clear band.

chimeric protein (without or with CT) revealed IL-4 but little or no type 1 cytokine response.

DISCUSSION

Lymphocytes taken from the PP and MLN of mice immunized i.g. with SBR or with SBR-CTA2/B without or with CT as adjuvant were capable of proliferating in vitro when stimulated with AgI/II, showing similar overall patterns of T-cell responses to the different regimens and stages of immunization as the serum and salivary antibody responses. Immunization with SBR alone induced the lowest proliferative responses in PP and MLN cells, and this was reflected also in the finding that there was little change in the proportions of $CD4^+$ and $CD8^+$ T cells in these organs. Moreover, the pattern of cytokine expression in the cells from PP and MLN of these mice suggested a mixed type 1 and type 2 helper activity, possibly arising from the different types of cells in the culture, or indicating regulation by Th0 cells (8). Coupling SBR to CTB in the form of the SBR-CTA2/B chimeric protein enhanced its immunogenicity with respect to T-cell responses in PP and MLN, and the addition of CT as an adjuvant further elevated these responses. Furthermore, the cytokine expression pattern in PP and MLN cells from mice immunized with SBR-CTA2/B (with or without CT) indicated that T-cell help was skewed toward Th2 activity. In part, these shifts might be explained by the enhanced migration of cells from PP to MLN and thence into the circulation and effector sites of mucosal immunity, but the decrease in Th1 cells in PP was not matched by a corresponding increase in MLN or spleen in this cross-sectional study. The finding of IL-5, IL-6, and IL-10 mRNAs in cell cultures regardless of antigen stimulation in vitro is not readily explained in terms of enhanced Th2 cell activity but may indicate constitutive expression of these cytokines or their continued expression ex vivo after immunization. It is also possible that IL-6 and IL-10 mRNAs were derived from macrophages present in the cell cultures, although these would be largely adherent and unlikely to be harvested along with the lymphocytes.

The proportions of $CD4^+$ T cells in PP increased after each additional dose of these immunogen preparations, but a corresponding increase was seen in MLN cells only from mice immunized with SBR-CTA2/B chimeric protein and CT adjuvant. The finding that these T-cell responses occurred in PP and MLN as early as after the first immunization, at least with SBR-CTA2/B, showed that antigen-sensitized T cells were elicited before IgA antibody responses became elevated in the effector sites of mucosal immunity such as salivary glands. The responses in MLN and PP were different, as significant proliferative responses and increased proportions of $CD4^+$ cells during the course of immunization were developed in MLN cells only when CT was used as an adjuvant, and moreover, MLN from all mice contained higher proportions of T cells of both phenotypes than corresponding PP. The proportion of $CD8^+$ cells was higher in MLN than in PP, but as it was not reduced by the administration of CT as an adjuvant, it appears that the enhanced AgI/II-specific proliferation in MLN cells from mice given CT is not due to inhibition of $CD8^+$ suppressor cells by CT (10). The spleen, a nonmucosal lymphoid organ, displayed little or no response in terms of antigen-specific proliferating T cells, despite the considerable elevation of serum IgG antibodies especially when SBR-CTA2/B was given together with CT adjuvant. This finding is consistent with the relatively modest numbers of specific antibody-secreting cells found in the spleen after i.g. immunization with AgI/II chemically conjugated to CTB and given with CT (36, 43). It is noteworthy that throughout these experiments, although the mice were immunized with SBR or SBR-CTA2/B chimeric protein, which represents residues 186 to 577 of AgI/II, both antibody and T-cell responses could be detected with intact AgI/II. This finding implies that SBR retains sufficient conformational structure similar to that of the corresponding part of the whole AgI/II molecule and that both are processed similarly by antigen-presenting cells.

These responses are in accordance with the concept of the common mucosal immune system and the dissemination of antigen-sensitized T and B cells from the inductive sites such as PP through the MLN that drain the lymph flow from the small intestine and thence into the circulation prior to relocation in the effector sites of mucosal immunity, including the salivary glands (27). Thus, i.g. immunization with SBR, especially when coupled to CTB in the form of a chimeric protein, leads to the appearance of antigen-responsive T cells in both PP and MLN. Because few cells were recoverable from blood, it was not practically possible to trace the appearance of such cells in the circulation, although this has been well documented in humans (1, 38), and the transient circulation of specific antibody-secreting cells, predominantly of the IgA isotype, approximately 1 week after mucosal immunization has been demonstrated in human and animal systems (3, 19, 35, 36). Curiously, perhaps, it appears that the peak of circulating antigen-specific T cells occurs after the peak of circulating antibody-secreting cells (1), and in the present experiments, an increased proportion of $CD4^+$ T helper cells was found in the peripheral blood of mice 10 days after the second or third dose of SBR-CTA2/B, especially if CT was also given as an adjuvant. Cytokine-secreting T cells are known to occur in effector sites of mucosal immunity,

such as the intestinal lamina propria and salivary glands (15, 32).

CT has been shown to enhance T helper responses in intestinal tissues, and particularly the response of the Th2 subset that is held to promote high levels of serum IgG and mucosal IgA antibody responses (17, 25, 29, 39, 44). We have likewise found evidence of type 2 cytokine production by antigen-specific T cells in nasal passage-associated lymphoid tissue and the draining cervical lymph nodes of mice immunized intranasally, as well as in PP and MLN of mice immunized i.g., with AgI/II conjugated to CTB (41, 42). CT has also been reported to deplete selectively $CD8^+$ intraepithelial lymphocytes (10), and while the functions and migratory potential of these cells are incompletely understood, any such effect within inductive sites such as the PP would also serve to elevate the proportion of $CD4^+$ T cells. However, in this study, although the proportion of $CD8^+$ cells declined slightly in some tissues, this decrease appeared to occur concomitantly with an increase in the number of $CD3^+$ cells, in particular the $CD4^+$ subset. Whether CTB itself can serve as an adjuvant in the absence of intact CT has been controversial. Synergism between CTB and CT has been demonstrated (23, 37, 40), and most commercially available, nonrecombinant preparations of CTB contain small amounts of intact CT that may be sufficient to show this effect. Working with chemical conjugates of AgI/II and CTB delivered i.g., we previously demonstrated that even with nonrecombinant CTB, it was necessary for the antigen to be coupled to CTB and for intact CT to be coadministered (4, 36). However, both this study and our previous reports (11, 13) show that the genetically constructed SBR-CTA2/B chimeric protein, in which the toxic CTA1 subunit has been deleted, is clearly able to induce mucosal and circulating antibodies without the necessity for additional CT. Although the adjuvant activity of CT may be closely linked to its toxicity, which is a function the ADP-ribosyltransferase activity of the A1 subunit (24), recent reports suggest that adjuvanticity of the related *E. coli* heat-labile enterotoxin can be dissociated from toxicity (6, 7). Fusion proteins of CTB directly coupled to other antigenic peptides have been constructed, but the conformation of CTB and its ability to form G_{M1}-binding pentamers tend to be disrupted by peptides longer than approximately 12 amino acid residues (5, 31), and moreover, their mucosal immunogenicity seems to be limited in the absence of additional CT. These limitations do not apply to SBR-CTA2/B chimeric protein, in which a large 42-kDa segment of protein is fused to the CTA2 subunit, which couples it noncovalently to the CTB pentamer to preserve its G_{M1} ganglioside-binding activity. The enhanced enteric immunogenicity of SBR-CTA2/B chimeric protein, even in the absence of CT, is advantageous for an oral vaccine, as recombinant CTB has been shown to be a safe and effective immunogen in humans (18).

We therefore conclude that i.g. immunization with SBR, especially when genetically coupled to CTB to enhance both mucosal and circulating antibody responses, induces T-cell responses in the gut-associated lymphoid tissues such as PP and MLN. Furthermore, these T-cell responses occur after one or two doses of immunogen, earlier than the antibody responses, and include increased proportions of $CD4^+$ T helper cells. The responses are enhanced by, but are not dependent on, the addition of CT as an adjuvant.

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Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins

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Escherichia coli heat-labile enterotoxin and cholera toxin are potent mucosal immunogens and adjuvants in animal models. Non-toxic mutants retaining adjuvant activity are useful tools to dissect the mechanism of mucosal adjuvanticity and promising candidates for development of human vaccines and immunotherapy. Clinical trials are expected to proceed in the near future.

The mucosal surfaces of the body are constantly exposed to a myriad of benign foreign antigens that are acquired through eating, breathing and touching, among others. Superficially, environmental or food antigens appear to be substantially ignored by the healthy immune system, which regards them as harmless. Indeed, we might actually show measurable immunological tolerance to them[1]. Relatively few molecules are highly immunogenic when they contact mucosal surfaces, in the sense that they generate strong humoral and secretory antibody responses. Such molecules are often referred to as mucosal immunogens.

The most powerful mucosal immunogens that are recognized to date are cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT), the molecules that cause the debilitating watery secretions typical of cholera and traveller's diarrhoea, respectively[2,3]. The mucosal immune system somehow recognizes that these toxins are a threat and, a short time after they make contact with a mucosal surface, a powerful immune response is mounted against them. This antitoxin response is so potent that sometimes a strong and easily measurable immune response is also activated against foreign bystander molecules that are present simultaneously at the mucosal surface[4].

As a consequence of this immunopotentiating property, CT and LT have been investigated extensively and exploited as mucosal immunogens and adjuvants in animal models[5,6]. However, the high toxicity of CT and LT makes them unsuitable for practical human use[2], thus prompting recent efforts to dissect the mucosal immunogenicity and adjuvanticity of CT and LT from their toxicity. Site-directed mutagenesis, guided by the crystal structure of the molecule and coupled with molecular modelling[7], has clarified our understanding of what makes these molecules so special in terms of mucosal immunity by disclosing the role of the receptor-binding domain, the B subunit, the A subunit and the enzymatic activity of LT and CT (Ref. 8). Critically, the use of highly purified recombinant material has also clarified compromised observations made previously with toxin-contaminated LT and CT B subunit derivatives (LTB and CTB, respectively)[8–10].

The relationship between structure and function of LT and CT

CT and LT belong to the AB class of bacterial toxins[11]. The two molecules have high homology (80% identity) in their primary structure[12,13] and superimposable tertiary structures[14]. Both toxins are composed of a pentameric B oligomer that binds the receptor(s) on the surface of eukaryotic cells, and an enzymatically active A subunit that is responsible for the toxicity (Fig. 1).

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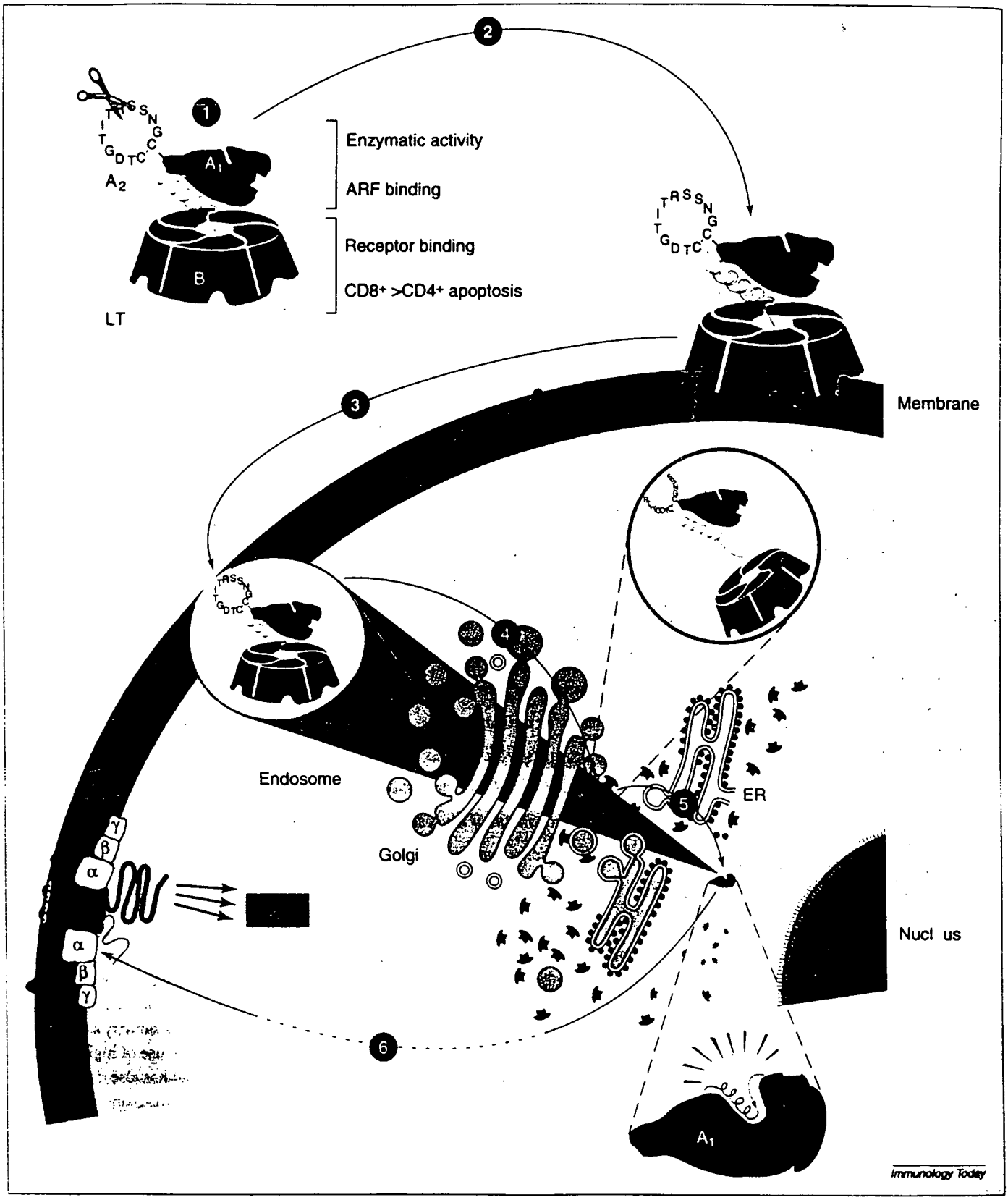
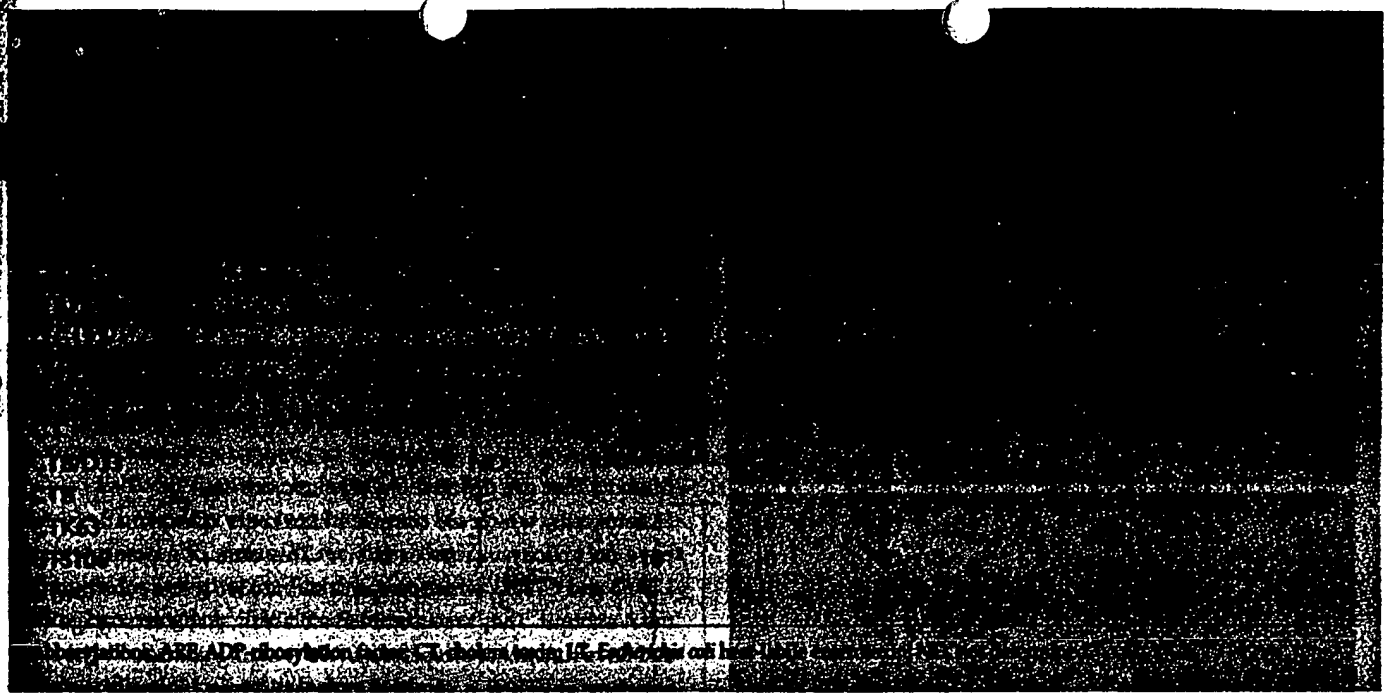


Fig. 1. *The sequence of events during cell intoxication. (1) Schematic representation of heat-labile enterotoxin (LT), showing the A and B subunits, their structural and functional significant features and the site of proteolytic cleavage of the loop between the A_1 and A_2 domains. (2) LT binds the receptor located on the plasma membrane of eukaryotic cells. (3) LT is internalized into vesicles. (4) The vesicles are transported to the Golgi apparatus where the holotoxin is disassembled. (5) The A subunit is transported from the Golgi to the endoplasmic reticulum (ER), the A or the A_1 subunit is translocated from the ER to the cytosol, where it can interact with the soluble ADP-ribosylation factor (ARF). (6) The activated A_1 migrates to the plasma membrane where the substrate G_s is located. The ADP-ribosylation of the α subunit of G_s induces permanent activation of adenylate cyclase and intracellular accumulation of cAMP. Abbreviations: Adc, adenylate cyclase; cAMP, cyclic AMP.*



The B oligomer

The B oligomer is a pentameric molecule of 55 kDa, containing five identical polypeptide monomers. The structure is compact, trypsin-resistant and requires boiling in the presence of sodium dodecyl sulphate to be dissociated. The five subunits are arranged in a cylinder-like structure, with a central cavity that exposes, on one side, five symmetrical cavities that are responsible for binding to the eukaryotic cell receptor[14] (Fig. 1). The receptor binding site is specific for a variety of galactose-containing molecules and shows a different fine specificity between LT and CT. CT binds mostly to the ganglioside GM1, which is believed to be the major toxin receptor[15], whereas LT binds not only GM1 (Ref. 16) but also other glycosphingolipids[17], glycoprotein receptors present in the intestine of rabbits and humans[18,19], polyglycosilceramides (PCGs)[20] and paragloboside[17]. Furthermore, the two variants of LT, human LT (hLT) and porcine LT (pLT), which differ by only four amino acids[21], are identical in their binding to glycoproteins and PCGs, but different in binding to paragloboside; pLT but not hLT binds paragloboside[20]. The different receptor binding activities of the LT and CT might be significant for the qualitatively different immunological properties that are exhibited by the two molecules, as will be discussed later.

Overall, two properties are associated with the B oligomer: (1) the ability to bind the receptor; and (2) to induce apoptosis of $CD8^+$ cells and, to a lesser extent, $CD4^+$ T cells (Fig. 1).

The A subunit

The A subunit is composed of a globular structure linked to the B oligomer by a trypsin-sensitive loop and a long α helix, the C-terminus of which enters into the central cavity of the B oligomer, thus anchoring the A subunit to the B pentamer[14]. Following protease cleavage of the loop, the A subunit is divided into the globular (enzymatically active) A_1 and the C-terminal A_2 fragments that remain linked by a disulphide bridge between the A_1–Cys187 and the A_2–Cys199 (see Fig. 1). Proteolytic cleavage of the loop and reduction of the disulphide bridge are both necessary for activation of the enzyme[22]. This loop is uncleaved when the molecules are produced in *E. coli*, however it is cleaved by a specific protease when molecules are produced in *Vibrio cholerae*[23]. The A_1 subunit contains an ADP-ribosylating enzymatically active pocket that binds nicotinamide adenine dinucleotide (NAD) and transfers the ADP-ribose group to the α subunit of several GTP-binding proteins that are involved in signal transduction. The consequences of transferral to G_s, the GTP-binding protein that regulates the activity of adenylate cyclase, are the best studied[11,24,25]. G_s ADP-ribosylation causes permanent activation of adenylate cyclase and abnormal intracellular accumulation of cAMP (Ref. 26; Fig. 1).

A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa GTP-binding proteins, known as ADP-ribosylation factors (ARFs). ARFs play a crucial role in vesicular membrane trafficking in both endocytic and exocytic pathways, and contribute to the maintenance of organelle integrity and assembly of coat proteins in eukaryotic cells[27]. Overall, the A_1 fragment can be seen as having at least two independent functions: enzymatic activity and ARF binding (Fig. 1).

The toxic sequence

The sequence of events that takes place during intoxication of eukaryotic cells[28–30] can be summarized as follows (see Fig. 1). The toxin binds the receptor and is internalized into vesicles that transport it to the Golgi compartment. Subsequently, the A and B subunits are dissociated, and the A subunit is transported from the Golgi to the endoplasmic reticulum (ER), whereas the B subunit persists in the Golgi and is later degraded. The A_1 subunit is then translocated from the ER to the cytosol, where it can interact with the soluble ARF and be activated. Finally, the A_1 subunit ADP-ribosylates the α subunit of G_s and possibly other G proteins located on the plasma membrane.

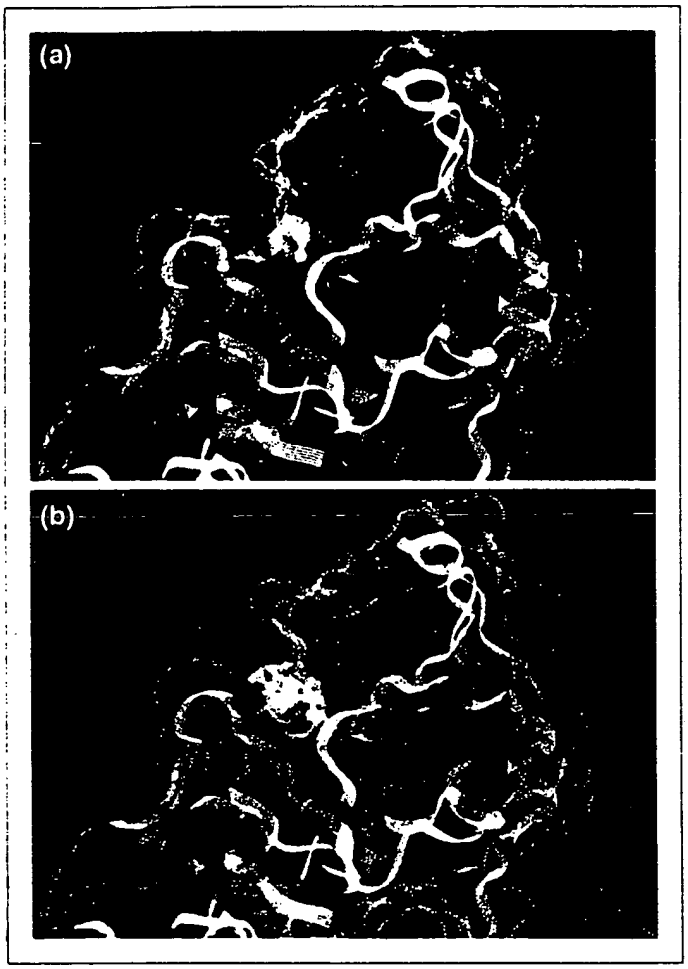


Fig. 2. *Three-dimensional structure of the A subunits of (a) wild-type heat-labile enterotoxin (LT) and (b) LTK63 represented as α-carbon trace and as solvent-exposed surfaces. The α helix and the β strand that form the nicotinamide adenine dinucleotide (NAD)-binding site are highlighted in red; the residues at position 63 [serine in the wild-type LT (a) and lysine in the LTK63 mutant (b)] are shown in yellow. The large, charged side-chain of the lysine in position 63 fills the NAD-binding cavity, thus obstructing it.*

Mutant toxin production and evaluation

To study the structure–function of CT and LT as well as to define molecules that are non-toxic but still active as mucosal adjuvants and immunogens, more than 50 different site-directed mutants have been produced[8–10,31–39]. The best characterized and most relevant are described below (see Table 1).

Mutants of the B subunit

Independently expressed B subunits were the first non-toxic derivatives of CT and LT to be produced. An oral vaccine against cholera, which contains recombinant CTB as a component, has been developed and thoroughly evaluated in animals and humans[40]. In this vaccine, CTB seems to act only as an immunogen and not as an adjuvant. Initial commercial preparations of CTB, in which the B subunit was purified from the active toxin, were associated with adjuvant activity; however, much of this activity was derived from contamination with active CT (Refs 41, 42). LTB seems to have a small variable but detectable adjuvant effect[8,10,43].

A mutant in LTB, defective in receptor binding (LTB/D33, containing a glycine to aspartic acid substitution at position 33), is non-immunogenic at mucosal surfaces, suggesting that an intact receptor binding site is necessary both for binding and immunogenicity[44]. Whether LT mutants that are defective in receptor binding, with and without enzymatic activity, are still active as adjuvants is controversial[37,45]. Non-binding LTB mutants also lose other immune-modulating activities, including their ability to induce apoptosis of $CD4^+$ and $CD8^+$ cells[44,46].

Mutants that are deficient in enzymatic activity

Holotoxoids, which are complete knockouts of enzymatic activity, have no toxicity *in vitro* or *in vivo*. This class of mutants includes LTK63 and CTK63, which contain a serine 63 to lysine substitution in the A subunit. They are assembled efficiently, stable on storage[32–34,47] and have functional receptor and ARF-binding domains[48]. The X-ray structure of LTK63 is identical to the wild-type LT across the entire molecule, with the exception of the active site where (as shown in Fig. 2) the bulky side chain of lysine 63 fills the catalytic cavity, thus making it unsuitable for enzymatic activity[49]. LTK63 is an excellent mucosal adjuvant, although the activity is reproducibly reduced in comparison with LT (Refs 8, 10, 50–53; Table 1; Fig. 3a), whereas CTK63 is a poor adjuvant[10] (Table 1; Fig. 3a). This adjuvant activity has been demonstrated using a wide range of antigens, including model antigens such as ovalbumin and protective antigens from bacterial and viral pathogens. Interestingly, LTK63 is consistently a better immunogen than LTB (Refs 8, 33, 39), suggesting an important role for the enzymatically inactive A subunit in the induction of an immune response. This is a property that might reflect not only the larger number of B- and T-cell epitopes provided by the A subunit but also its ability to influence intracellular events, such as antigen processing and presentation. In addition, the poor adjuvanticity of CTK63 has been associated with poor immunogenicity[10]. Differences between the adjuvant activity of LTK63 and CTK63 might reflect the effect of the different receptor binding affinities of these proteins. Other CT holotoxoid mutants that are described in the literature as mucosal adjuvants include CTF61 and CTK112 (containing serine 61 to phenylalanine and a glutamic acid 112 to lysine substitutions, respectively). However, it is important to point out that in those experiments, the amount of CT mutants used was ten times higher than that of wild-type CT (Ref. 38). These controversial results will be discussed later.

A further class of mutant molecules contains LTR72 (with an alanine to arginine substitution in position 72 of the A subunit) and CTS106 (with a proline to serine substitution in position 106 of the A subunit). These mutants have approximately 1% of the wild-type ADP-ribosylating activity, *in vitro* toxicity in Y1 cells reduced by a factor of 10^4–10^5 and approximately 1% toxicity *in vivo* (Fig. 3b). Both LTR72 and CTS106 are excellent mucosal adjuvants, being as effective as LT and CT, respectively[8,10] (Table 1; Fig. 3a). These two mutant holotoxoids might have their toxicity reduced sufficiently for safe use in humans, although still maintaining a little enzymatic activity, which significantly enhances their adjuvanticity.

Mutants in the protease-sensitive loop

Mutants in this region were constructed to make the loop insensitive to proteases and hence eliminate the susceptibility of the toxin to the cleavage required for activation of the enzymatic activity and toxicity. The best characterized mutant is LTG192, in which arginine 192 is replaced by a glycine[35,54,55]. *In vitro*, the mutant is completely trypsin-resistant; however, *in vivo*, proteases other than trypsin can cleave the loop and activate the toxin, as toxicity is detectable. The toxicity observed in Y1 cells is approximately 10^3 times lower than the wild-type toxin during the first 8 h of incubation, becoming only 5–10 times lower than wild-type following longer incubation[54]. In practice, this molecule takes longer to be activated but delivers approximately the same total enzymatic activity as wild-type. The difference is that the delivery of the active toxin is diluted over a longer period of time. *In vivo*, in the rabbit ileal loop, very little difference in toxicity is observed between LTG192 and wild-type LT (Ref. 56; Fig. 3b). Ongoing human trials are expected to establish the safety profile of this molecule[56]. LTG192 is indistinguishable from wild-type toxin both in terms of immunogenicity and adjuvanticity (Table 1; Fig. 3a).

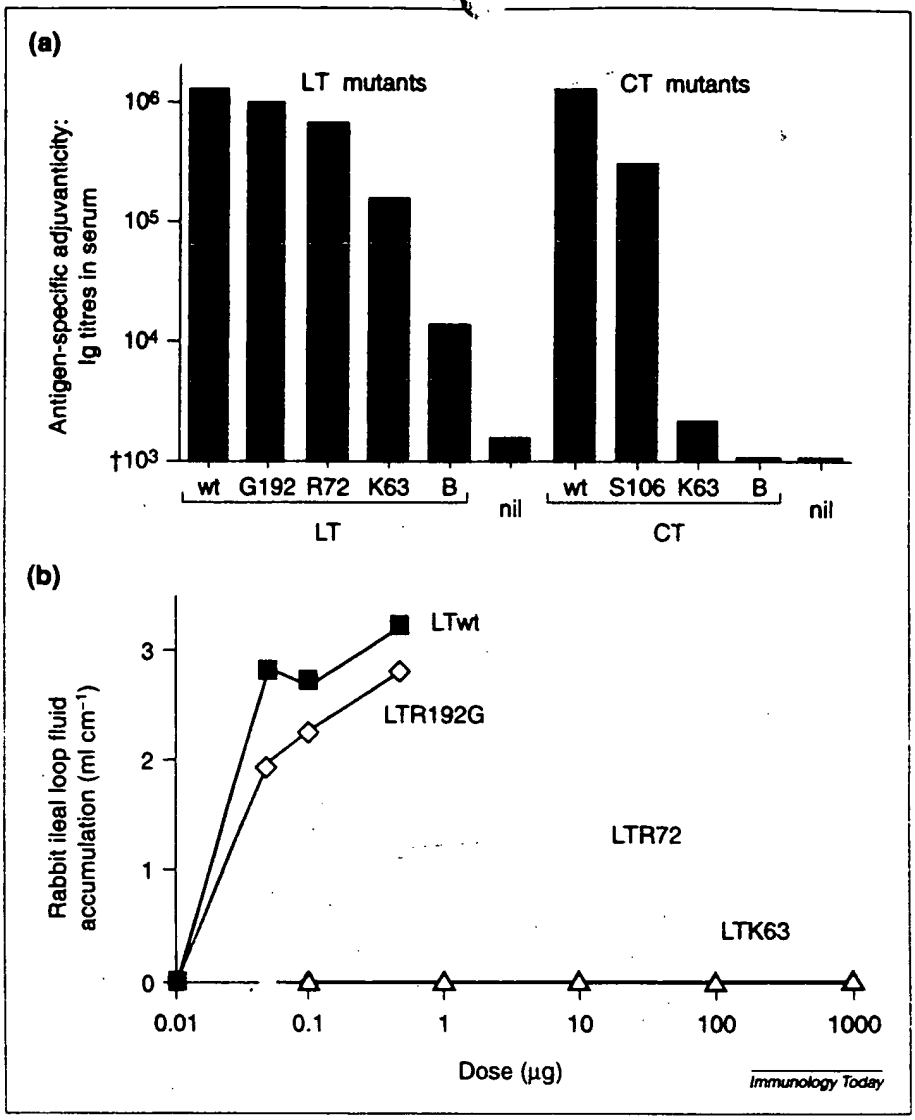


Fig. 3. *(a) Immunoglobulin (Ig) immune response to bystander antigens in sera of mice immunized intranasally with wild-type (wt) cholera toxin (CT) and heat-labile enterotoxin (LT) and their genetically detoxified derivatives as adjuvants. Results are shown as mean titres of antigen-specific antibodies. (b)* In vivo *toxicity in the rabbit ileal loop assay. Toxicity is expressed as the fluid accumulation (ratio of the amount of fluid collected in each loop to the length of the loop) induced by different amounts of LT and its genetically detoxified derivatives.*

Recombinant A subunit

An alternative approach to separate the adjuvant activity of LT and CT from toxicity has been the use of the A subunit alone. The A subunit of CT has been expressed as a fusion protein with a B-cell targeting moiety and the two Ig-binding domains (DD) of staphylococcal protein A, and hence called CTA1–DD fusion protein. This molecule retains the adjuvant activity of CT by directing the enzymatic activity of the A subunit to B cells and possibly other antigen-presenting cells[57]. Enzymatically inactive CTA1–DD derivatives fail to induce an adjuvant response following systemic immunization showing that the adjuvant effect of CTA1–DD depends on the enzymatic activity[58]. However, whether the enzymatic activity of these fusions is also essential for mucosal adjuvanticity has not yet been investigated.

In other experiments, both the His-tagged form of LTA, and the His-tagged form of the enzymatically inactive derivative LTA-K112 [LTA$(His)_{10}$ and LTA-K112 $(His)_{10}$, respectively] have been reported to retain the mucosal adjuvant properties of the wild-type toxin, suggesting that in this case the adjuvant effect is independent from ADP-ribosylation[37]. The mechanism by which a His-tagged A subunit can be internalized in the absence of a receptor binding domain is unclear. It is possible that the polycationic histidine peptide tail could provide a non-specific cell binding activity[59].

Non-toxic mutants act also as oral adjuvants

Most of the results so far described were obtained in mice by using the intranasal route for immunization. This route is very convenient because immunogenicity and adjuvanticity can be induced by using as little as a fraction of a microgram of antigen and adjuvant. Whether the conclusions so far reached are valid also for the oral route remains unresolved in the literature. Oral immunization usually requires large amounts of antigen (100–5000 μg) and adjuvant (at least 50 μg), and therefore most experiments are technically compromised by the potential involuntary intranasal contamination with small fractions of the vaccine during oral immunization. A second factor especially critical for oral adjuvanticity is the structural

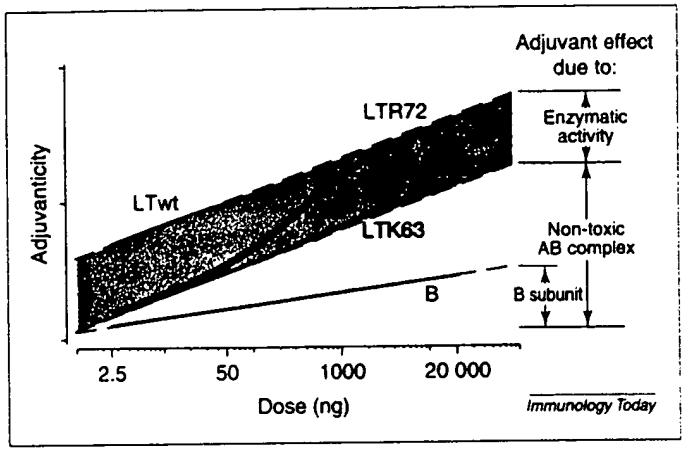


Fig. 4. *The results of a dose-response experiment that show the relative contribution of the B subunit, the non-toxic AB complex and the enzymatic activity to adjuvanticity.*

stability and protease sensitivity of both the mutants and the bystander antigen. This can influence the *in vivo* persistence of the holotoxin structure and therefore its activity. Recent studies taking into account the above technical problems, have come to the conclusion that LT, LTG192 and the non-toxic LT derivative LTK63, act as oral adjuvants to varying degrees. The B subunit alone is less successful as an adjuvant via this route[60].

Effects on the immune system

The literature describing the behaviour of LT and CT on the mucosal immune system is extensive and in many places contradictory. CT-mediated adjuvanticity appears to be accompanied by a preferential activation of T helper 2 (Th2)-type $CD4^+$ cell populations. This comes from the observation that mucosal immunization with antigens plus CT induces increased production of interleukins (IL) 4, 5 and 10, the predominant production of immunoglobulin G1 (IgG1) isotype and induction of antigen-specific IgE (Ref. 6). More recently, this polarization of the immune response towards a Th2 functional phenotype has been shown to be caused by the ability of CT to inhibit both the production of IL-12 p70 and the expression of the β1 and β2 chains of the IL-12 receptor. This might lead to the functional suppression of Th1 cell differentiation and to polarization towards a Th2-type response[61]. The ability of CT to polarize the immune response towards Th2 and to induce a selective upregulation of the B7-2 expression[62] is also maintained by enzymatically inactive mutants such as CTK112 (Ref. 63).

This polarization in the T-cell response is much less pronounced when LT is used as a mucosal adjuvant, with both Th1 and Th2 cells being activated[5]. In addition, recent studies suggest that LT mutants with one single amino acid substitution in the A subunit have different behaviours in the activation of the $CD4^+$ cell subpopulation. In these studies, the fully non-toxic LTK63 mutant promoted T-cell responses with a mixed Th1–Th2 profile, whereas the LTR72 mutant, which retained residual enzymatic activity, induced a more polar-

The polarization of the immune response seems to be affected not only by the different adjuvant molecules used, but also by the route of immunization[64].

Conclusions

Binding to mucosal receptors is a danger signal

Receptor binding is necessary for LT and CT to induce a mucosal immune response. This suggests that receptor binding is sufficient to differentiate LT and CT from the thousands of other molecules that associate with the mucosal surfaces without inducing a similar response. This observation is likely to be generally applicable to every molecule.

Mucosal surfaces provide a physical barrier between the external environment and the body, and the substances that come into contact with the mucosae usually do not closely interact with them. Exceptions are the small molecules that interact with the receptors for odours and taste and the small-molecular-weight peptides that are taken up in the gut following digestion of the proteins present in the food. Therefore, it makes sense for the body to mount a vigorous immune response against every molecule that actively binds to mucosal surfaces. In fact, this might be a signal that a molecule is trying to behave abnormally and therefore is potentially dangerous. Several reports that describe the mucosal immunogenicity of proteins that bind to mucosal receptors support the above conclusion. However, binding to the mucin layer of the gut rather than receptors on cells might not be sufficient to induce an immune response. Hence, some molecules with binding activity might not actually reach immune inductive sites or might be presented inappropriately, resulting in them being treated as environmental.

The surprising finding that some enzymatically inactive mutants are good mucosal adjuvants, whereas other mutants are not, can be explained by several factors: (1) their stability *in vivo*; (2) the efficiency of the ER targeting retention sequence (KDEL for CT and RDEL for LT)[65,66]; and (3) the different specificity of the receptor-binding site. The differential binding of LT and CT to different receptors might target the two molecules to different cell populations, thus changing their adjuvant effect. Because the receptors might be present in different tissues and in different animal species, we should consider the data reported here as relating only to intranasal delivery in the mouse. Therefore, it should not be too surprising if future studies will show CT mutants to be better adjuvants than LT mutants in different animal species or if delivered by different routes.

The non-toxic AB complex and enzymatic activity in adjuvanticity

The availability of molecularly defined mutants has enabled the relative contributions of the B subunit, the non-toxic AB complex and the enzymatic activity to adjuvanticity to be studied. A dose response curve comparing LTK63, LTR72, wild-type LT and LTB as adjuvants showed that the B subunit is a poor adjuvant at all doses (although some adjuvanticity is present at very high doses), whereas

that is dose-dependent (Fig. 4). By contrast, the enzymatic activity provides a dose-independent adjuvant effect above a certain threshold of activity. This threshold is reached at 2.5 ng with LT and at approximately 1 μg with LTR72.

The adjuvant activity of the non-toxic AB complex might derive from properties of the A subunit, such as the binding to ARF factors or the ability to interact with the vesicular transport system. This could allow antigens present in LT-containing endosomes to reach the Golgi and the ER, thus facilitating their interaction with antigen-presenting systems. The marked dose dependence of the adjuvant activity of enzymatically inactive derivatives of LT and CT is enough to explain most of the controversial results present in the literature. Adjuvanticity can be demonstrated for virtually every molecule by increasing the dose used. To make sure that the best mutants are finally selected for human use and that scientifically sound conclusions are reached, similar doses should be compared in future studies.

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Eotaxin: from an eosinophilic chemokine to a major regulator of allergic reactions

Jose Carlos Gutierrez-Ramos, Clare Lloyd and Jose Angel Gonzalo

Eotaxin has a variety of effects on several cell types that are involved in the allergic inflammatory response. Here, Jose Carlos Gutierrez-Ramos and colleagues review the chemotactic effects of eotaxin on eosinophils and T helper type 2 cells, its differentiation and migration effects on mast cells and its actions on progenitors and mature cells in the bone marrow.

The elegant identification of eotaxin, a peptide with eosinophil chemotactic activities[1,2] opened the door to our understanding of the molecular basis for the highly selective eosinophil accumulation seen during allergic reactions. The molecular cloning of the gene encoding eotaxin[3–5], and the subsequent study of its sequence revealed that it belonged to a family of peptide chemoattractants termed chemokines. The chemokines are peptide ligands for the seven-transmembrane G-protein-coupled receptors expressed on leukocytes, which elicit changes in adhesiveness, cell motility and chemotaxis[6]. Based on function and sequence homologies, it was evident that eotaxin belonged to the CC subfamily of chemokines[3–5]. The CC subfamily of chemokines, which includes monocyte chemoattractant proteins (MCPs), RANTES and macrophage inflammatory proteins (MIPs) among others, acts predominantly on monocytes, lymphocytes and non-neutrophil granulocytes (reviewed in Ref. 6).

The protein product of the eotaxin gene was shown to be a very potent and efficacious chemoattractant for eosinophils *in vivo* and *in vitro*[3–5]. It is present and its synthesis regulated during allergic reactions and other pathological processes in which eosinophils are thought to play a role[7–11]. These clues were expeditiously followed by several groups, whose efforts resulted in the cloning of the gene encoding the eotaxin receptor, CCR3 (Refs 12, 13).

The identification of a chemotactic factor for eosinophils and its receptor was only the beginning of a series of findings that raised eotaxin to a unique position among the players in allergic inflammation

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Rapid note

Immunostimulatory activity of LT-IIa, a type II heat-labile enterotoxin of *Escherichia coli*

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Abstract

Certain bacterial molecules potentiate immune responses to parenterally administered antigens. One such molecule that has been intensely investigated is cholera toxin, a type I heat-labile enterotoxin produced by the Gram-negative bacterium *Vibrio cholerae*. Immunization with a mixture of a foreign antigen and cholera toxin enhances the immune response to the antigen. Similar adjuvant activity is associated with LT-I, a closely related type I heat-labile enterotoxin produced by *Escherichia coli*. The adjuvant activities of LT-IIa, a member of the type II heat-labile enterotoxins produced by *E. coli*, have not been described. LT-IIa and CT differ significantly in amino acid sequence of the B polypeptides and in receptor binding affinity. In this study, rats were subcutaneously immunized with fimbrillin, a protein isolated from the bacterium *Porphyromonas gingivalis*, and with fimbrillin in combination with LT-IIa, the prototypical type II enterotoxin. Previous studies documented that fimbrillin administered alone is a poor immunogen. Animals immunized with the mixture of fimbrillin and LT-IIa produced high titers of specific IgG antibody directed against fimbrillin. Anti-fimbrillin antibody titers in sera from animals receiving the combination of LT-IIa + fimbrillin were comparable to those obtained from sera of animals immunized with cholera toxin + fimbrillin. The results of these experiments demonstrate that LT-IIa exhibits an adjuvant activity that is equal to that of cholera toxin. Recombinant methods have been established for producing large amounts of LT-IIa, an advantage that will likely provide an economic impetus to consider incorporating the enterotoxin as an immunostimulatory agent in future vaccines.

Keywords: Adjuvant; Enterotoxin; Fimbrillin; LT-IIa

The heat-labile enterotoxins produced by *Escherichia coli* and *Vibrio cholerae* belong to a family of proteins that are related in structure and function [1]. Members of this family include cholera toxin (CT) expressed by *V. cholerae* and the enterotoxins LT-I and LT-IIa, expressed by enterotoxigenic strains of *Escherichia coli* [2]. All members of the family are oligomeric proteins, composed of a single A subunit that is non-covalently bound to a pentameric array of B polypeptides [2]. Treatment of holotoxin with trypsin cleaves the A polypeptide into two fragments joined by a disulfide bond [3]. The A1 fragment is a potent ADP-ribosyl transferase that is responsible for the enterotoxigenic effects, while the A2 fragment derived from the C-terminal end of the A polypeptide non-covalently binds the A subunit to the B pentamer. The B pentamer mediates binding of the enterotoxin to gangliosides [4], a heterogeneous family of sugar-containing lipids on the surface of mammalian cells [5]. Each enterotoxin

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has a unique ganglioside-binding specificity [4]. CT binds to ganglioside GM1 (GM1), whereas LT-I binds to GM1 and several glycoproteins [6]. LT-IIa binds with strong affinity to ganglioside GD1b (GD1b), and with less affinity to gangliosides GD1a, GM1, GT1b, GQ1b, and GD2 [4]. The property to recognize different receptors likely enables the enterotoxins to bind to the surface of different cells or tissues [7].

CT, LT-I and LT-IIa have been purified and the operons encoding the A and B subunit genes have been cloned and sequenced (reviewed in [2]). Comparisons of the predicted amino acid sequences show that the A polypeptides of CT, LT-I, and LT-IIa are similar (at least 50% identity) [2]. In contrast, the B polypeptides of CT and LT-I, while highly homologous to each other (at least 80% identity), have little or no homology to the B polypeptides of LT-IIa (less than 14% identity). The differences in amino acid sequences are sufficient to produce antigenic heterogeneity between the members of the family. Antisera against CT and LT-I will not react with LT-IIa, and vice versa [2]. Based on differences in immunoreactivity, the family of heat-labile enterotoxins was divided into two classes, the type I and type II enterotoxins. Type I enterotoxins include CT and LT-I, while LT-IIa, and a related enterotoxin, LT-IIb, comprise the type II class of heat-labile enterotoxins.

CT is a highly immunogenic protein that stimulates potent secretory and systemic immune responses [8,9] and is known to have adjuvant activity. Both secretory and humoral immune responses to foreign antigena are enhanced when an animal is immunized with a mixture of antigen and CT [10]. Whereas adjuvant activity is most pronounced when CT holotoxin is employed, it has been demonstrated that immunization with the non-toxic B pentamer also elicits an enhanced immune response to foreign antigens [10,11]. These studies suggest that the immunomodulatory properties of CT are associated, in part, with GM1-binding activity.

While the adjuvant activity of CT has been established, the potential of the type II heat-labile enterotoxin LT-IIa for enhancing immune responses has not been investigated. We hypothesized that with its binding affinity for a variety of gangliosides, LT-IIa may bind to and stimulate cells of the immune system that are not bound or stimulated by CT or may bind to the same cells in larger quantities. In either case, we predicted that the adjuvant activity of LT-IIa, if present, would be equal or greater than that of CT.

To determine if LT-IIa has adjuvant activity and to compare that potential adjuvant activity with that of CT, conventional Sprague–Dawley rats were immunized with fimbrillin in the presence or absence of purified CT or LT-IIa (a gift from Dr R.K. Holmes). Fimbrillin is the major polypeptide comprising the fimbrial structure of the Gram-negative oral pathogen *Porphyromonas gingivalis* [12]. Parenteral immunization with fimbriae elicits only a weak anti-fimbrillin antibody response in the rat model [13]. For these experiments, five groups of rats ($n = 4–8$ rats per group) were immunized with purified fimbrillin [13], CT, LT-IIa, CT + fimbrillin, or LT-IIa + fimbrillin. Rats ($n = 6$) that received sham immunizations of buffer without enterotoxin or fimbrillin served as a control group. Solutions used for immunizations contained enterotoxin and fimbrillin at concentrations of 10 μg/ml and 100 μg/ml, respectively. One hundred microlitres of the immunizing solutions were administered subcutaneously at each of two sites. The amounts of enterotoxin (2 μg) and antigen (20 μg) used were comparable to amounts of antigen used in similar immunization studies [7,13]. Phosphate-buffered saline (PBS) was used as the diluent in all cases. Rats were immunized at day 0 and at day 28 with enterotoxin, fimbrillin, or enterotoxin + fimbrillin, as appropriate. Forty-two days after the primary immunization, animals that were immunized with fimbrillin, CT + fimbrillin, and LT-IIa + fimbrillin received a second booster immunization of fimbrillin. The remaining groups (CT, LT-IIa, and the control group) received sham immunizations of PBS at day 42. A blood sample was collected from the lateral tail vein of each rat prior to the initial immunization (day 0) and at days 14, 28, 42 and 49. Serum was separated from the clotted blood by centrifugation.

Particle concentration fluorescence immunoassay (PCFIA) was used to measure the amount of anti-fimbrillin antibodies in serum samples [14]. In brief, purified fimbrillin was covalently bound to carboxylated polystyrene beads (IDEXX, Westbrook, ME) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St. Louis, MO). Titration studies were used to determine the amount of fimbrillin that was required in the binding reaction to saturate the surface of the beads with the antigen (data not shown). Fimbrillin-coated beads were incubated with a 1:100 dilution of serum, washed with PBS to remove unbound antibody, and incubated with a 1:35 dilution of fluorescein isothiocynate (FITC)-labeled affinity purified goat anti-rat IgG immunoglobulin (Kirkegaard and Perry, Gaithersburg, MD). After extensive washing to remove unbound FITC-labeled antibodies, fluorescence of the treated beads was measured at 485/535 nm in an automated fluorimeter (IDEXX, Westbrook, ME). Anti-fimbrillin antibody titers in the sera were expressed as relative units of fluorescence (RFU). Previous studies demonstrated that the level of fluorescence detected by PCFIA is directly correlated with the amount of antibody bound to the antigen-sensitized beads [13]. The technique is highly quantitative [13]. The amount of antibodies in the serum samples directed against LT-IIa and CT was measured by an enzyme-linked immunosorbent assay (GD1b-ELISA) [15]. Rat hyperimmune

antiserum raised against purified fimbrillin was used as a positive control in all experiments. Data from different groups were compared using an analysis of variance test to determine significance.

As expected, rats immunized with CT + fimbrillin showed a much stronger response to the fimbrial antigen than did rats immunized with fimbrillin alone ($P < 0.001$) (Fig. 1). By day 42, the anti-fimbrillin response elicited by immunization with fimbrillin was meager (4072 ± 559 RFU) (data are reported as the mean RFU $\pm$ one standard error of the mean). Rats immunized with CT + fimbrillin responded strongly on day 42 and produced high titers of anti-fimbrillin antibody (13372 ± 1539 RFU). Seven days after a booster immunization with fimbrillin (day 49), the immune response to the antigen was enhanced further in the CT + fimbrillin group (35698 ± 2757 RFU). Animals boosted with fimbrillin alone showed a small enhancement in specific antibody responses (13782 ± 2743 RFU), but the enhancement was not as dramatic as with the animals that had been previously immunized with fimbrillin + CT or fimbrillin + LT-II.

Analysis of the sera from rats immunized with fimbrillin and LT-IIa + fimbrillin demonstrated that LT-IIa has potent adjuvant activity (Fig. 1). By day 42, rats immunized with LT-IIa + fimbrillin developed a significantly higher titer of anti-fimbrillin antibody than

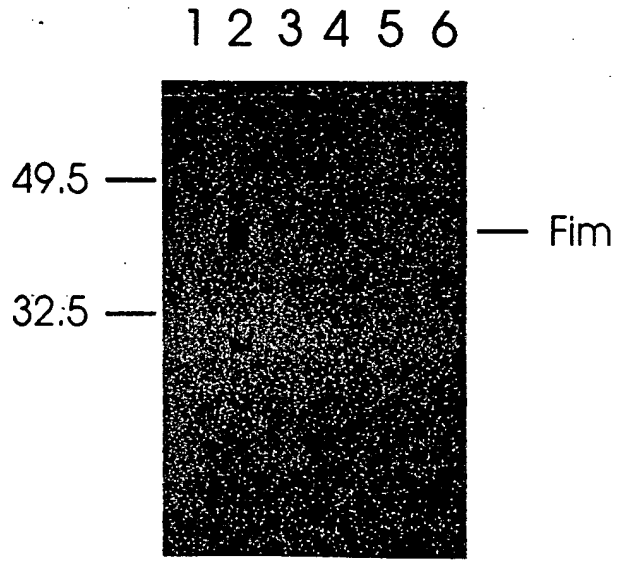

Fig. 1. Immunoblot analysis of the enterotoxin-enhanced immune response to fimbrillin. Purified fimbrillin was immunoblotted with a 1:50 dilution of antisera collected on day 49 from rats immunized with: 1, CT; 2, CT + fimbrillin; 3, LT-IIa; 4, LT-IIa + fimbrillin; 5, fimbrillin; and 6, PBS (sham immunization). In each case, the antiserum for the immunoblot was obtained from a rat in the relevant group that had a specific antibody titer that most closely approximated the mean antibody titer of the group, as determined by PCFIA. Immunoblots were developed using goat anti-rat IgG (BioDesign, Kennebunk, MA) and 4-chloro-1-napthol (Biorad, Richmond, CA).

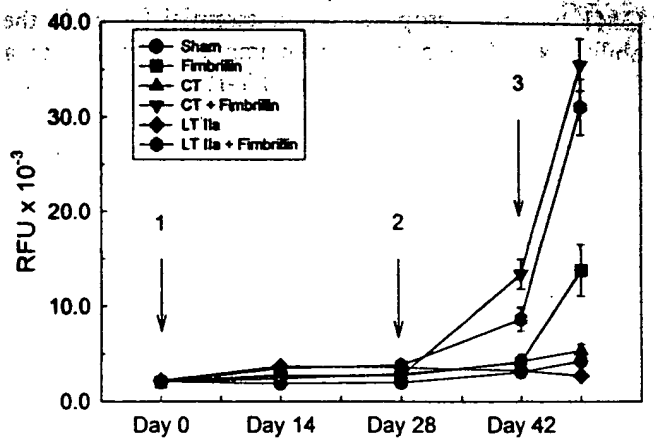

Fig. 2. Enhanced IgG-specific immune response to fimbrillin after co-immunization with CT or LT-IIa. Rats were immunized with fimbrillin, LT-IIa, CT, fimbrillin + CT, and fimbrillin + LT-IIa. Symbols for each group are noted in the box. IgG antibody titers in the sera collected on days 0, 14, 28, 42 and 49 were measured by PCFIA. Time points at which immunizations were given are denoted by arrows: 1, initial immunization; 2, second immunization; 3, booster immunization using purified fimbrillin (given only to groups previously immunized with fimbrillin, fimbrillin + CT, or fimbrillin + LT-IIa). RFU—relative fluorescence units. Bars indicate one standard error of the mean.

did rats immunized with fimbrillin without the enterotoxin (8644 ± 1242 RFU vs 4072 ± 559 RFU, respectively; $P < 0.001$) (Fig. 1). By day 49 after booster immunizations, the differences between the two groups increased (31226 ± 2969 vs 13782 ± 2743 RFU, respectively; $P < 0.001$). Comparisons of the levels of antibodies produced by rats immunized with CT + fimbrillin to the levels of antibodies produced by rats immunized with LT-IIa + fimbrillin showed that the two groups of rats had equal anti-fimbrillin antibody titers ($P = 0.31$).

To confirm that the immunoreactivity in the serum samples detected by PCFIA was directed against fimbrillin, pooled sera from each group were diluted 1:50 and analyzed by Western blotting (Fig. 1). Sera collected on day 49 from rats immunized with fimbrillin + CT and with fimbrillin + LT-IIa showed a strong response to purified fimbrillin. Although sera from rats immunized with fimbrillin without enterotoxin had detectible immunoreactivity by PCFIA, the amount of anti-fimbrillin antibody in a 1:50 dilution was below the level of detection of the immunoblot (Fig. 2). The rats in all groups immunized with enterotoxins produced high levels of anti-enterotoxin antibodies. Sera collected from animals in the CT and the CT + fimbrillin group had high titers of CT-specific antibodies and sera from animals in the LT-IIa and LT-IIa + fimbrillin groups had high titers of anti-LT-IIa antibodies (data not shown).

The data demonstrate that LT-IIa and CT are equally potent adjuvants in this rat model. Affinity for

GM1 has been proposed as an essential factor in the ability of CT to potentiate an immune response to a foreign antigen [11]. Although LT-IIa binds to GM1, GM1 is not the preferred receptor [4]. It is not clear whether the adjuvant activity of LT-IIa is a result of low affinity binding of the enterotoxin to GM1 or to high affinity binding of the enterotoxin to alternative gangliosides on host cells or tissues involved in immune responsiveness. Mutant LT-IIa enterotoxins with altered ganglioside-binding specificities and mutant LT-IIa enterotoxins with no detectible ganglioside-binding activities have been reported [15]. It will be interesting to employ these mutant toxins in experiments similar to those reported here to determine in a direct manner the importance of ganglioside-binding in induction of adjuvant activity.

This report establishes that LT-IIa is a potent immunostimulatory molecule. Further studies of LT-IIa must be done to define the factors that are most important in potentiating immune responses. The ability to produce very large amounts of LT-IIa using established recombinant methods [15] should provide an economic impetus for the use of the enterotoxin in the development and production of future vaccines.

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Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of *Escherichia coli* heat labile enterotoxin in attenuated salmonellae

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Two overlapping T-cell sites of the nucleocapsid antigen (HBc) of Hepatitis B Virus (HBV) (amino acids (aa) 120–140) and a B-cell epitope of the pre-S(2) region of the HBV surface antigen (aa 133–140) were expressed as a fusion protein with the subunit B of* Escherichia coli *heat labile enterotoxin (LT-B) in attenuated salmonellae (*aroA Salmonella dublin *SL1438). When Balb/c (haplotype H-2^d) mice were fed salmonellae expressing LT-B or the LT-B/HBV fusion protein they developed serum IgG anti-LT-B antibodies and splenic cells reactive to LT-B. C57BL/10 (H-2^b), in contrast, showed anti-LT-B antibody titres, but no splenic cell priming to LT-B. Neither in Balb/c nor in C57BL/10 mice could an antibody response to the fused HBV antibody binding site be demonstrated. In C57BL/10, however, an HBc T-cell epitope fused to LT-B primed a splenic cell response to an analogous synthetic peptide (HBc aa 121–145) in four out of five mice after three oral immunizations. This is the first description of the priming of a cellular immune response to a defined heterologous epitope expressed in attenuated salmonellae and delivered by the oral route.

Keywords: Hepatitis B; T-cell epitope; fusion protein; *E. coli* enterotoxin

Introduction

Infection with hepatitis B virus (HBV), a small DNA virus (for reviews see refs 1, 2, 3) is the cause of acute and chronic liver disease in man and HBV continues to be one of the most important pathogens on a world-wide scale with over 200 million chronic carriers[4]. Despite the existence of safe and efficient plasma-derived and recombinant vaccines[5], availability and cost still hamper vaccination programmes similar to the successful immunization against smallpox virus. Existing vaccines are based on HBV surface antigens. Immunization with recombinant HBV core antigen (HBc) has also been shown to provide some degree of protection against HBV infection[6,7], probably mediated by cellular immune response mechanisms, since HBc is an internal viral antigen. HBc particles elicit both T-cell dependent and T-cell independent antibody responses and a strong cellular immune response[8]. T-cell epitopes of HBc have been mapped in the murine system[9], but it is not known which epitopes mediate protective immunity. It has recently been demonstrated that immunization with recombinant (human) HBc induces a partial protection in woodchucks against infection with a closely related hepadnavirus, woodchuck hepatitis virus (WHV) (F. Schödel, K. Manneck, K. Fuchs, H. Will and M. Roggendorf, unpublished). Therefore HBc amino acid sequences mediating protective immune responses are probably in the regions conserved among WHV and HBV (for a comparison of amino acid sequences see Ref. 1). An ideal vaccine would comprise protective T-cell epitopes for a longlasting memory and elicit virus neutralizing antibodies against defined epitopes. It would be an additional advantage if this vaccine could be given orally and would combine stability and ease of production. Invasive but non-pathogenic *aroA* salmonella strains have been developed[10,11] which confer immunity to salmonella infections and are suitable as carriers for heterologous antigens (Refs 10, 12; for a review see Ref. 13). It has been shown that oral immunization with recombinant salmonellae expressing a circumsporozoite antigen can induce protective, possibly cell-mediated, immunity against *Plasmodium berghei* infection in mice[14]. Antibodies against HBV surface antigen epitopes could be elicited by the same route when the epitopes were expressed in salmonellae as flagellin inserts[15]. The development of an expression system which allows the stable expression of foreign epitopes as fusion proteins with the subunit B of *Escherichia coli* heat labile enterotoxin (LT-B) was described previously[16]. These fusion proteins expressed in SL1438, an *aroA Salmonella dublin* auxotroph, induce high level serum IgG antibodies to LT-B when fed orally to Balb/c or C57BL/10 mice and splenic T-cells reactive with LT-B when fed to Balb/c[17]. Serum antibodies to the fused HBV antibody binding sites could not be detected. It was described

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recently that two HBc T-cell epitopes (HBc amino acids (aa) 120–140) fused to an antibody binding site of the middle HBV surface protein in a synthetic peptide elicited both a strong H-2 restricted T-cell response after immunization in mice and provided T-cell help for the attached B-cell epitope (HBV pre-S(2) amino acids 133–140)[18]. It has been possible to stably express the same HBc T-cell epitopes and the B-cell epitope as a fusion protein with the carboxy terminus of LT-B (expression plasmid pFS20) in the vaccine strain SL1438[17]. Here the cellular immune response in mice fed with SL1438(pFS20) against one of the two HBc T-cell epitopes and against LT-B, is described.

Materials and methods

Bacteria and plasmids

The *aroA S. dublin* vaccine strain SL1438[10] was kindly provided by Bruce Stocker (Stanford). The construction of expression plasmids pFS2.2 (expressing LT-B + eight carboxyterminal amino acids from a polylinker) and pFS20 (HBc aa 120–145 and HBV pre-S(2) aa 133–140, fused to the carboxyterminus of LT-B) has been described previously[17]. Maintenance and growth conditions of bacteria were as described before[16,17].

Immunization

As described previously[16,17] 10–12 female Balb/c or C57BL/10 mice were fed with $\approx 10^{10}$ colony forming units (c.f.u.) of SL1438(pFS20) [group M, C57BL/10 and group N, Balb/c] or SL1438(pFS2.2) [group O] by orogastric tube. Blood was drawn 10 days after the first immunization and the animals were immunized a second time. Again blood was drawn after 10 days a set of animals was killed and the spleens were removed for establishing T-cell cultures. A second set of animals was immunized a third time and blood and spleen cells removed 14 days after the third immunization.

Cell culture and antibody determination

Spleens were aseptically removed and carefully dissected. Splenic cells were separated over a Ficoll/Isopaque gradient and cultured in RPMI-Click (Gibco) supplemented with 0.5% syngeneic serum in microtitre plates. The cell density was adjusted to 5×10^5 cells/well and affinity purifed LT-B (kindly provided by John Clements) to a concentration of 0.1 μg, 1 μg or 10 μg ml^{-1} or a synthetic peptide spanning HBc amino acids 121–145 (synthesized by automated synthesis, >90% pure by HPLC analysis) to a concentration of 1 μg, 10 μg or 100 μg ml^{-1} was added. The HBc synthetic peptide was weighed, solubilized by ultrasound, sterile filtrated and diluted before use. The indicated concentrations reflect the amount of peptide weighted in, actual concentrations in the cell culture wells may be lower due to low solubility of the peptide and loss during sterile filtration. The splenic cells were cultivated in a humid atmosphere at 37°C and 5% CO_2 for 5 days. In the last 18 h of culture, the cells were labelled with 0.5 μCi ^{3}H-thymidine (Amersham), harvested and counted in a liquid scintillation cocktail (Rackbeta). Background values of cells cultivated in the absence of antigen were determined and automatically subtracted. Anti-LT-B and anti-HBV pre-S(2) antibodies in the serum were determined at a 1/100 serum dilution by GM1-ELISA and anti-peptide (HBV pre-S(2) aa 133–150) ELISA, respectively, as described[17]; the second antibody was goat anti-mouse IgG (H + L), peroxidase coupled (Medac, Hamburg), substrate orthophenyldiamine.

Results

The cellular and humoral immune responses of mice fed with salmonellae expressing LT-B or a LT-B/HBV fusion protein were analysed. As previously demonstrated for LT-B alone and other LT-B fusion proteins[17], mice fed with salmonellae expressing either LT-B or the LT-B/HBc/HBV-pre-S(2) fusion protein developed serum IgG antibodies against LT-B (*Figure 1, a–c*). In Balb/c

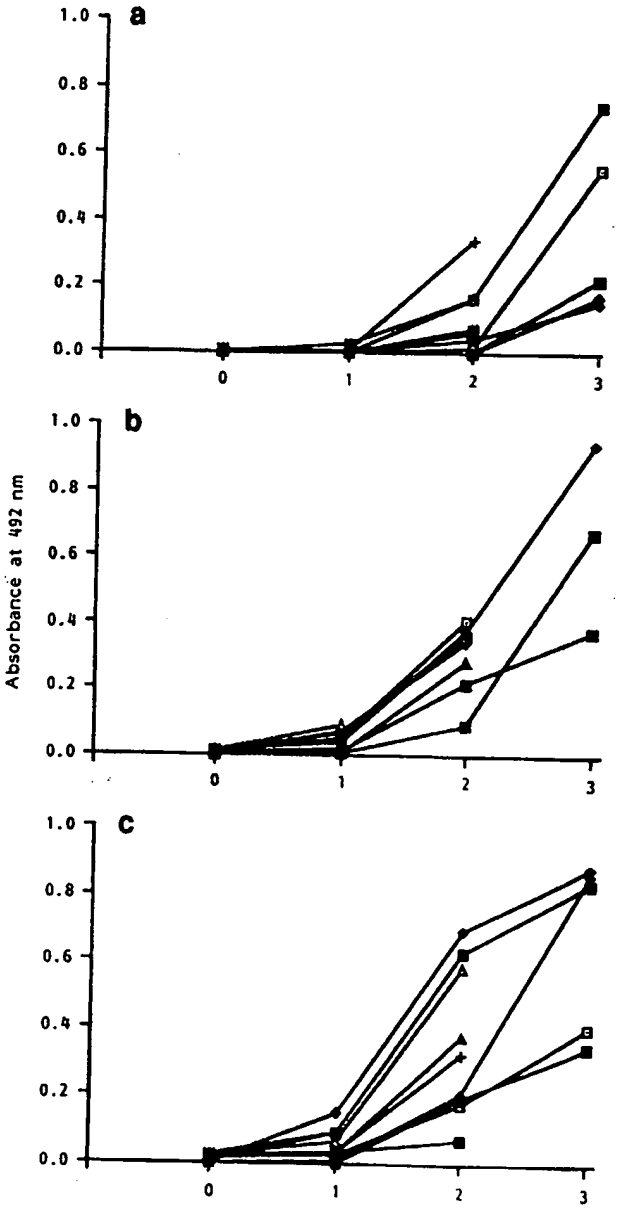
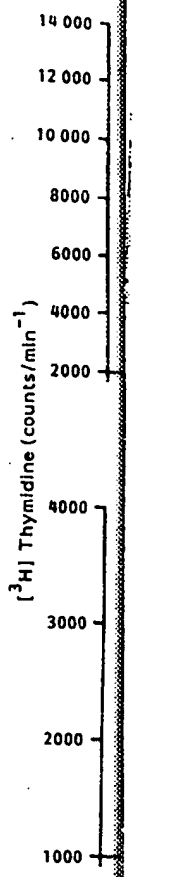


Figure 1 Serum IgG anti-LT-B antibodies after oral immunization of C57BL/10 mice with SL1438 (pFS20) (a), or Balb/c mice with SL1438(pFS20) (b) or SL1438(pFS2.2) (c). The means of duplicate absorbance measurements at 492 nm in a GM1 ELISA are indicated: 0 = pre-immune, 1 = 10 days after the first immunization, 2 = 10 days after the second immunization, 3 = 14 days after the third immunization. Lines connect values of individual sera



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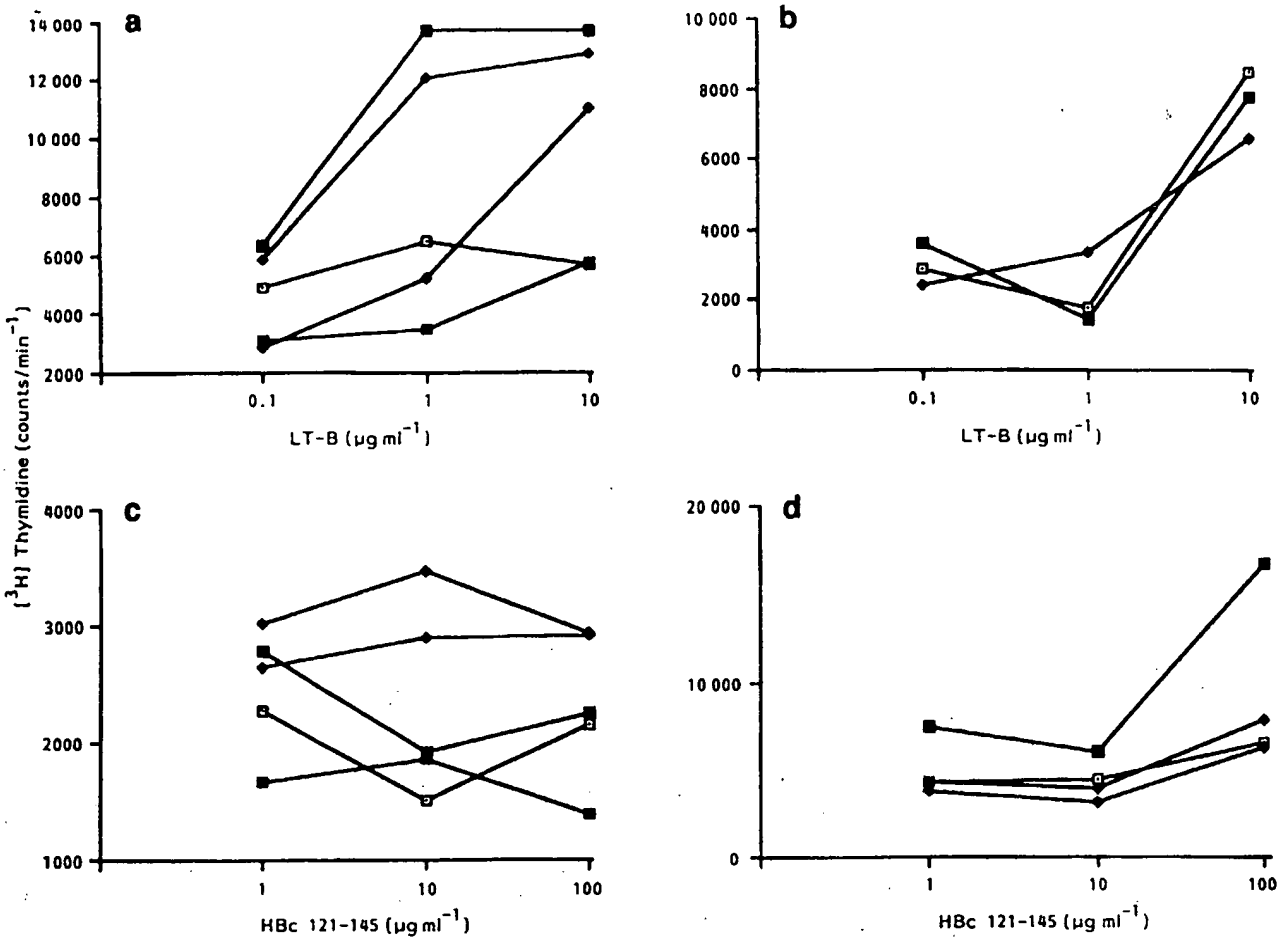

Figure 2 Stimulation of splenic cells in the presence of LT-B (a,b) or a synthetic peptide from the HBc region aa 121–145 (c,d). Mean counts min^{-1} of triplicate cultures labelled with ^{3}H-thymidine are indicated. Values of individual animals are connected. (a), group N (Balb/c, SL1438(pSF20)), (b) and (c), group O (Balb/c, SL1438(pFS2.2)), (d), group M (C57BL/10, SL1438(pFS20), here only the values of four out of five animals showing thymidine incorporation are plotted

mice, a higher rate of seroconversion was observed after the second oral immunization (*Figure 1b,c*) than in C57BL/10 mice (*Figure 1a*). After the third immunization all animal sera analysed showed detectable levels of serum anti-LT-B IgG. Antibodies against the carboxyterminally fused HBV-pre-S(2) B-cell epitope could not be detected in either Balb/c or C57BL/10 mice by anti-peptide ELISA (data not shown). After the third oral immunization, splenic cells reactive to LT-B could be observed in Balb/c mice fed with LT-B or LT-B fusion protein expressing salmonellae (*Figure 2a,b*). In contrast, in C57BL/10 mice no splenic cells primed against LT-B were detected (data not shown). Apart from a possible major histocompatibility complex restriction for LT-B this may be a hint that LT-B is capable of inducing antibodies by a T-cell independent mechanism, as antibodies against LT-B were readily detected in C57BL/10. In Balb/c an LT-B dose dependent increase in ^{3}H-thymidine incorporation was observed whether the animals were immunized with salmonellae expressing LT-B alone (group O, *Figure 2b*) or the fusion protein (group N, *Figure 2a*). The stimulation seems, however, higher in the group that received salmonellae expressing only LT-B (*Figure 2b*). Only in mice with an H-2^b genctic background (C57BL/10) was a stimulation of splenic cells in the presence of the HBc analogous synthetic peptide observed (*Figure 2d*); in Balb/c mice (H-2^d) SL1438(pFS20) did not prime a response (not shown). The HBc 121–145 synthetic peptide induced no proliferative response in splenic cells of Balb/c fed with SL1438(pFS2.2) as a negative control (*Figure 2c*). Similarly, no *in vitro* proliferative response to the synthetic peptide was observed in splenic cells of unprimed C57BL/10 or of C57BL/10 fed with salmonellae expressing LT-B alone (not shown). This observation is consistent with the previously described H-2 restriction of the HBc T-cell epitope 129–140[9,18]. Primed T cells of the HBc synthetic peptide could be demonstrated in four out of five mice analysed after the third immunization with SL1438(pFS20).

Discussion

The data presented indicate that a heterologous T-cell epitope fused to the carboxyterminus of LT-B can be presented to the mouse immune system by orally given attenuated salmonella. To our knowledge this is the first time that it has been demonstrated that attenuated salmonella can induce an immune response to a defined heterologous T-cell epitope by the oral route. Heterologous B-cell epitopes fused to the carboxyterminus of LT-B, although they are accessible on the non-denatured protein, as tested by ELISA type assays with the respective poly- or monoclonal antibodies (data not

shown), do not induce a detectable B-cell response. The reason for the low B-cell immunogenicity of the fused sequences compared with LT-B alone is not known. It has recently been demonstrated that B-cell epitopes of the holera toxin subunit B, highly homologous to LT-B, and of the HBV pre-(S) and S region inserted into a hypervariable sequence of H-1d phase-1 flagellin can be expressed in *aroA S. typhimurium* in an immunogenic form[15,19] and in the case of the HBV epitopes induce low serum antibodies by the oral route after four subsequent immunizations in a mouse model[15]. This approach could lead to the formation of neutralizing antibodies against HBV in man provided that the epitopes can be expressed in suitable *S. typhi* in an immunogenic form. Induction of T-cell responses to one or two T-cell sites may not suffice to induce protection in an outbred population. As the T-cell sites used here (HBc120–140) are particularly well conserved between HBV and WHV[1] they could play a role in inducing protective immunity. The finding reported here that a T-cell response can be induced by orally given *Salmonella* vaccine strains together with the successful induction of antibody responses to heterologous flagellin inserts expressed in salmonellae[15] paves the way for construction of recombinant bacteria that present a selection of artificially combined T-cell epitopes together with B-cell epitopes eliciting neutralizing antibodies, possibly on different structures within the same bacterium. Since the amount of heterologous antigen actually delivered to the host immune system may be critically affected by plasmid loss *in vivo* in the experiments described here and the presence of antibiotic resistance markers on plasmids used for *in vivo* vaccinations is not desirable, we have constructed *asd* plasmids expressing LT-B and LT-B/HBV fusion proteins and are currently testing their immunologic properties (F. Schödel, H. Will, T. Doggett and R. Curtiss, unpublished results).

These expression plasmids without antibiotic resistance markers are highly maintained in salmonella vaccine strains by complementation of a lethal mutation in the cell wall metabolism (Δ aspartate semialdehyde dehydrogenase [*asd*])[20]. Cells that lose the plasmids lyse and release their antigenic content. Also hybrid HBc/pre-S proteins which form particles[21] have now been successfully expressed in attenuated salmonellae (F. Schödel, unpublished results) and their immunogenicity is under investigation.

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The authors are grateful to Bruce Stocker for providing SL1438 and SL5283, to John Clements for purified LT-B and to Makoto Mayumi for anti-HBV pre-S(2) monoclonal antibodies. The expert technical assistance of Ursula Morgenroth is gratefully acknowledged. Work in the authors' laboratory was in part supported by grants of the Wilhelm Sander and the Walter Schulz foundations to FS.

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Characterization of Hybrid Toxins Produced in *Escherichia coli* by Assembly of A and B Polypeptides from Type I and Type II Heat-Labile Enterotoxins

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The genes encoding the individual A and B polypeptides of the type I enterotoxin LTp-I and type II enterotoxins LT-IIa and LT-IIb were cloned and tested for complementation in *Escherichia coli*. Each gene encoding an A polypeptide was cloned into pACYC184, and each gene encoding a B polypeptide was cloned into the compatible plasmid Bluescript KS+. In addition, operon fusions representing all combinations of A and B genes were constructed in Bluescript KS+. Extracts from strains of *E. coli* expressing each combination of A and B genes, either from compatible plasmids or from operon fusions, were tested for immunoreactive holotoxin by radioimmunoassays and for toxicity by Y1 adrenal cell assays. Biologically active holotoxin was detected in each case, but the toxicity of extracts containing the hybrid toxins was usually less than that of extracts containing the wild-type holotoxins. The ganglioside-binding activity of each holotoxin was tested, and in each case, the B polypeptide determined the ganglioside-binding specificity. The A and B polypeptides of the type II heat-labile enterotoxins were also shown to form holotoxin in vitro without exposure to denaturing conditions, in contrast to the polypeptides of the type I enterotoxins that failed to form holotoxin in vitro under comparable conditions. These findings suggest that type I and type II enterotoxins have conserved structural features that permit their A and B polypeptides to form hybrid holotoxins, although the B polypeptides of the type I and type II enterotoxins have very little amino acid sequence homology.

The heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* constitute a family of proteins that are related in structure and function (22, 33). They are classified into two serogroups (9, 21, 37–39). Antisera against the enterotoxins in serogroup I do not neutralize the toxins in serogroup II, and vice versa. Serogroup I includes cholera toxin (CT) and the *E. coli* type I heat-labile enterotoxins (LT-I). LTh-I and LTp-I are antigenically cross-reacting variants of LT-I produced by *E. coli* strains from humans and pigs, respectively (6, 23). Serogroup II includes the *E. coli* type II heat-labile enterotoxins (LT-II), with antigenic variants designated LT-IIa and LT-IIb (14, 15, 19, 21, 37–39). Strains of *E. coli* that produce LT-II have been isolated from animals (water buffalo, cattle, pigs, etc.) and foods, particularly in Southeast Asia and South America, but they are rarely isolated from humans (14, 40). CT and LT-I function as virulence factors and cause diarrhea with cholera and enterotoxigenic *E. coli* infections, but the role of type II toxins in pathogenesis has not yet been demonstrated.

Toxins in the *V. cholerae* and *E. coli* heat-labile enterotoxin family have one A polypeptide and five B polypeptides, and quaternary structure of the holotoxins is maintained by noncovalent bonds between the polypeptides (12, 14, 16, 22, 43). Treatment of holotoxin with trypsin cleaves its A polypeptide into fragments A1 and A2, which remain joined by a disulfide bond, and fragment A2 mediates interaction of the A polypeptide with the B polypeptides. Holotoxin binds via its B polypeptides to surface-exposed sugars of gangliosides that function as specific receptors on eucaryotic cell membranes (11, 22). Both CT and LT-I bind preferentially to ganglioside GM1, but LT-I can also bind to glycoproteins (13). LT-IIa binds with greatest affinity to ganglioside GD1b, and LT-IIb binds preferentially to ganglioside GD1a (11). Binding of holotoxin triggers entry of fragment A1 into the target cells, in which it activates plasma membrane adenylate cyclase by catalyzing ADP ribosylation of the regulatory protein $G_{s\alpha}$. Biological effects of the heat-labile enterotoxins are believed to be mediated primarily by increased concentrations of cyclic 3′,5′-AMP (cAMP) in the target cells, but cAMP-independent effects of enterotoxins that may be mediated by prostaglandins or eicosanoids or by cross-linking of gangliosides in plasma membranes have also been described previously (10, 35).

The genes that encode the A and B polypeptides of CT, LTh-I, LTp-I, LT-IIa, and LT-IIb have all been cloned and sequenced (7, 27, 28, 32, 36, 37, 39, 45). Comparison of the predicted amino acid sequences for these representative type I and type II enterotoxins shows that their A1 fragments are most homologous (22, 36–39, 45); this homology presumably reflects the conserved ADP ribosyltransferase activities of the A1 fragments (26). The A2 fragments are much less homologous than the A1 fragments (36, 38). Within serogroup I or II, the B polypeptides are homologous, but B polypeptides of type I enterotoxins have little or no significant homology with those of type II (36, 38). The apparent lack of homology between B polypeptides of type I and type II enterotoxins is consistent with their different ganglioside-binding specificities, and the limited homology between A2 fragments of type I and type II enterotoxins may reflect coevolution of the A2 polypeptides with the specific B polypeptides to which they bind (22).

Assembly of type I holotoxins occurs after transport of their A and B precursor polypeptides into the periplasm and removal of their signal peptides (7, 16, 17, 32). The mature B polypeptides of type I enterotoxins can associate to form

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TABLE 1. Strains and plasmids used in this study

Plasmid or *E. coli* strain	Phenotype	Construction	Reference
Vectors and parental plasmids			
pACYC184	Nontoxinogenic	Clr^r Tet^r, P15A replicon	5
pBluescript	Nontoxinogenic	Amp^r, ColE1 replicon, P_{lac} (Stratagene)	41
pEWD299	LT-I A, LT-I B	LT-I operon from p307	7
pCP3727	LT-IIa A, LT-IIa B	LT-IIa operon from *E. coli* SA53	39
pCP4185	LT-IIb A, LT-IIb	LT-IIb operon from *E. coli* 41	31
pACYC184 constructions			
pTC900	LT-I A	1.3-kbp *Sma*I fragment from pEWD299	This study
pTC304	LT-IIa A	1-kbp *Pst*I fragment from pCP3727	This study
pTC601	LT-IIb A	1.58-kbp *Eco*RV-*Dra*I fragment from pCP4185	This study
pBluescript constructions			
pTC801	LT-I B	750-bp *Hin*dIII fragment from pEWD299	This study
pTC400	LT-IIa B	800-bp *Eco*RI-*Hpa*I fragment from pCP3727	This study
pTC700	LT-IIb B	1.0-kbp *Hin*dIII-*Pst*I fragment from pCP4185	This study
pTC5000	LT-I A, LT-I B	1-kbp *Bam*HI-*Eco*RI fragment from pTC900 and 3.6-kbp fragment from pTC801	This study
pTC200	LT-IIa A, LT-IIa B	2.2-kbp *Eco*RI-*Kpn*I fragment from pCP3727	This study
pTC101	LT-IIb A, LT-IIb B	1.68-kbp *Bgl*II-*Hpa*I fragment from pCP4185	This study
pTC1000	LT-I A, LT-IIa B	1.3-kbp *Cla*I-*Sal*I fragment from pTC900 into pTC400	This study
pTC2000	LT-I A, LT-IIb B	1.3-kbp *Cla*I-*Sal*I fragment from pTC900 into pTC700	This study
pTC3000	LT-IIa A, LT-I B	1-kbp *Bam*HI-*Eco*RV fragment from pTC304 into pTC801	This study
pTC4000	LT-IIb A, LT-I B	1.6-kbp *Cla*I-*Bam*HI fragment from pTC601 into pTC801	This study
Strains			
XL1-Blue	Nontoxinogenic	*recA1 supE44 relA1 lac* (F' *proAB lacIq* Z M15 Tn*10*)	4
SA53	LT-IIa A, LT-IIa B	Parental strain expressing LT-IIa toxin	14
41	LT-IIb A, LT-IIb B	Parental strain expressing LT-IIb toxin	14
T1	LT-IIa A, LT-IIa B	XL1-Blue containing pTC304 and pTC400	This study
T2	LT-IIb A, LT-IIb B	XL1-Blue containing pTC601 and pTC700	This study
T3	LT-IIa A, LT-IIb B	XL1-Blue containing pTC304 and pTC700	This study
T4	LT-IIb A, LT-IIa B	XL1-Blue containing pTC601 and pTC400	This study
T5	LT-I A, LT-I B	XL1-Blue containing pTC900 and pTC801	This study
T6	LT-I A, LT-IIa B	XL1-Blue containing pTC900 and pTC400	This study
T7	LT-I A, LT-IIb B	XL1-Blue containing pTC900 and pTC700	This study
T8	LT-IIa A, LT-I B	XL1-Blue containing pTC304 and pTC801	This study
T9	LT-IIb A, LT-I B	XL1-Blue containing pTC601 and pTC801	This study

pentamers in vivo or in vitro, either in the presence or absence of mature homologous A polypeptides, but interaction with homologous A polypeptides increases the rate of association of B polypeptides and favors formation of holotoxin instead of B pentamers (16). Assembled B pentamers of type I enterotoxins do not associate with A subunits to form holotoxin (16). Hybrids between CT and LT-I can be produced by denaturing and renaturing mixtures of their A and B polypeptides in vitro, and such hybrid type I enterotoxins have toxicity comparable to that of native CT or LT-I (16, 44).

In the current study, we cloned the genes encoding the A and B polypeptides of LTp-I, LT-IIa, and LT-IIb and tested all possible pairwise combinations of their A and B genes, either in compatible plasmid vectors or in hybrid operon constructs, for complementation in *E. coli*. Our goals were to determine whether heterologous A and B polypeptides from type I and type II enterotoxins can associate to form hybrid holotoxins and, if so, to characterize the hybrid holotoxins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The LTp-I-producing plasmid pEWD299 (8) and the plasmids pCP3727 expressing LT-IIa (37, 39) and pCP4185 expressing LT-IIb (38) have been described previously. Genes encoding individual toxin polypeptides were cloned into the vectors pBluescript KS+ (or KS−) (Stratagene) and pACYC184 (5) by using standard methods (30). Genes cloned into pBluescript vectors were oriented for expression under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter. *E. coli* XL1-Blue (Stratagene) was used as the host strain for all recombinants (4). Details of construction and phenotypes of the recombinant plasmids and strains used in this study are summarized in Table 1.

DNA procedures. DNA was isolated by using the Ish-Horowicz and Burke modification (24) of the alkaline extraction procedure of Birnboim and Doly (3). Agarose gel electrophoresis was performed by standard procedures (30), and restriction fragments used for cloning were isolated from gel slices by electroelution. Restriction enzymes and other DNA-modifying enzymes were used according to suppliers' specifications (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; and New England Biolabs, Beverly, Mass.). Transformations were performed by using $CaCl_2$-treated cells as described by Maniatis et al. (30).

Preparation of toxin-containing extracts. *E. coli* strains expressing cloned genes for toxin A and/or B polypeptides from recombinant plasmids were cultured in LB broth (42). Ampicillin (75 μg/ml) and/or chloramphenicol (25 μg/ml) was

added as appropriate to select for plasmid-determined resistance traits. Cultures were incubated at 37°C with vigorous shaking. IPTG was added (0.5 to 1.0 μg/ml) at mid-log stage (optical density at 600 nm of ~0.2 to 0.4), and incubation was continued overnight. Cells were pelleted by centrifugation and resuspended in 1/30 of the original volume of phosphate-buffered saline (PBS). Periplasmic toxins or polypeptides were released either by sonication or by treatment with polymyxin B sulfate (2 mg/ml for 30 min at room temperature) (25). Sonicates and extracts were centrifuged to pellet debris, and supernatants containing toxin or subunits were collected and used as described below. Extracts were either assayed and used immediately or stored at 4°C and reassayed before use in other experiments.

Assays for biological activity of heat-labile enterotoxins. Methods to test for biological activity of the toxin by using Y1 adrenal cells have been described previously (29). Samples (100 μl) containing serial dilutions of toxin extracts in RPMI 1640 were applied to monolayers of Y1 adrenal cells and incubated for 16 h. One unit of activity was defined as the minimum dose of toxin required to give a 4+ reaction, corresponding to rounding of at least 75% of the cells in the monolayer (29), and biological activity (toxicity) was expressed as units per milliliter. Extracts from XL1-Blue cells harboring pBluescript and/or pACYC184 plasmids were used as controls and were not toxic for Y1 cells. Purified LTp-I, LT-IIa, and LT-IIb toxins were used as positive controls. It is not possible to convert toxic activity of extracts directly to enterotoxin concentration, because specific activity depends on the ratio of nicked toxin to unnicked toxin. For the purified, fully nicked toxins, one toxic unit corresponds to approximately 12.5 pg of LTp-I (21), 0.5 pg of LT-IIa (21), and 3 pg of LT-IIb (15).

Solid-phase radioimmunoassays. Ganglioside-dependent solid-phase radioimmunoassays with monoclonal antibodies (MAb) directed against the A polypeptides of heat-labile enterotoxins were used to measure the amounts of immunoreactive holotoxin in extracts (20). A solution containing gangliosides GM1, GD1a, and GD1b (Matreya, Inc., Pleasant Gap, Pa.) at 0.8 μg of each per ml in PBS was prepared, and 25 μl of the mixed ganglioside solution (20 ng of each ganglioside) was added to each well of a 96-well polyvinyl microtiter plate (Dynatech, Alexandria, Va.). The plates were incubated at room temperature overnight, and unbound gangliosides were removed by washing the plates with PBS. The wells were then blocked by treatment for 30 min at room temperature with PBS containing 10% horse serum (PBS-10; GIBCO). All subsequent steps were performed at 37°C. Extracts containing toxins were serially diluted by using PBS-10, and 25-μl samples of each dilution were applied to the wells and incubated for 3 h. Wells were then washed with PBS containing 1% horse serum (PBS-1) and probed with 25 μl of hybridoma cell culture supernatant containing the appropriate mouse MAb specific for the A subunit present in the extract: MAb 4B6 (3.6 μg/ml) for LTp-I, MAb 5C5 (3.8 μg/ml) for LT-IIa, and MAb 19G5 (concentration not determined) for LT-IIb. The isotypes of MAb 4B6 and MAb 5C5 are immunoglobulin G1 (IgG1); that of MAb 19G5 is IgA. Details concerning the preparation and characterization of MAb 5C5 and MAb 19G5 will be described elsewhere (18a). None of these MAb cross-reacts with any of the enterotoxin B subunits. After 1 h of incubation, unbound antibodies were removed by rinsing with PBS-1. Wells were then probed with a 1:2,000 dilution of rabbit anti-mouse IgG (Sigma, St. Louis, Mo.) or rabbit anti-mouse IgA (Sigma), as appropriate, for 1 h, washed with PBS-1, and incubated for 90 min with ^{125}I-labelled goat anti-rabbit IgG (Sigma). After being washed with PBS-1, wells were cut from the plate, and the radioactivity of the ^{125}I-labelled goat anti-rabbit IgG bound to the well was counted by using a Micromedic Model MEplus Gamma Counter. Known amounts of purified LTp-I, LT-IIa, and LT-IIb toxins were used as standards, and immunoreactive holotoxin was expressed as micrograms of the standard enterotoxin containing the same A subunit as the extract per milliliter.

A modification of the procedure reported by Fukuta et al. (11) was used to measure the relative binding activities of hybrid toxins for different gangliosides. Polyvinyl microtiter plates were prepared as above, except that each well contained only one of the three gangliosides (GM1, GD1a, or GD1b) and the ganglioside was serially diluted in PBS in successive wells in a twofold series from 25 ng to 12 pg per well. Extracts were diluted to 200 ng of immunoreactive holotoxin per ml, and each extract was assayed on each of the three ganglioside-containing plates. The plates were incubated for 3 h and processed as described above.

Immunoadsorption of wild-type and hybrid holotoxins. To confirm that the biological activity of extracts containing homologous and heterologous toxin subunits was associated with holotoxin, Sepharose beads conjugated with streptococcal G-protein (Pharmacia, Inc., Piscataway, N.J.) were used to make specific immunoadsorbents. Aliquots (100 μl) of Sepharose–G-protein beads were incubated overnight with hybridoma supernatants containing MAb specific for the B subunits present in the extract to be adsorbed: MAb 15G3 for the B subunit of LT-IIa (63 μg of IgG per ml), MAb 20C9 for the B subunit of LT-IIb (24 μg of IgG per ml), and MAb 12G5 for the B subunit of LTp-I (70 μg of IgG per ml) (2). All three MAb are IgG1 isotypes. Details concerning the preparation and characterization of MAb 15G3 and MAb 20C9 will be described elsewhere (18a). None of these B-polypeptide-specific MAb cross-reacts with any of the toxin A polypeptides. After incubation, the beads were washed extensively to remove unbound MAb, and the beads were then used to adsorb 220 μl of toxin extracts which had been previously diluted to between 40 and 640 U/ml of biologically-active holotoxin. The titers of adsorbed and unadsorbed extracts were determined to ascertain the smallest dose of each that was cytotoxic in the Y1 adrenal cell assay. Solutions of purified LTp-I (873 pg/ml), LT-IIa (68 pg/ml), and LT-IIb (2.6 ng/ml) toxins were used as controls.

In vitro assembly of holotoxin from A and B subunits. To determine whether A and B subunits could assemble in vitro to form holotoxin, extracts from strains expressing the A or B subunit of LTp-I, LT-IIa, or LT-IIb were mixed in all pairwise combinations of A and B subunits. Equal volumes (10 to 50 μl) of A and B subunit extracts were combined, and the heterologous and homologous mixtures were incubated for 10 min at 37°C. Mixtures were then applied to microtiter plates sensitized with a mixture of GM1, GD1a, and GD1b gangliosides (20 ng of each per well) and measured for immunoreactive holotoxin by solid-phase radioimmunoassay using A-polypeptide-specific MAb, as described above. Samples containing only A or B subunit extracts were used as controls, and purified LTp-I, LT-IIa, and LT-IIb toxins were used as standards. To determine the effect, if any, of polymyxin B sulfate on assembly, sonicated extracts were also assayed. Polymyxin extracts and sonicated extracts behaved similarly in in vitro assembly experiments (data not shown). Similar mixtures of extracts containing A and B subunits were also measured by Y1 adrenal cell assays to determine whether cytotoxic holotoxin was produced. Con-

TABLE 2. Immunoreactivity and biological activity of heat-labile enterotoxins with homologous and heterologous A and B subunits encoded by structural genes on separate plasmids or in hybrid operons

Toxin composition		Activity of toxin subunits encoded by genes on:					
		Complementary plasmid			Operon fusion		
Subunit A	Subunit B	Biological activity[a] (U/ml)	Toxin antigen[b] (μg/ml)	Sp act (U/μg)	Biological activity (U/ml)	Toxin antigen (μg/ml)	Sp act (U/μg)
LT-IIa	LT-IIa	27,000	5.1	5,270	656,000	80.0	8,200
LT-IIb	LT-IIb	32,000	87.5	370	23,300	13.6	1,790
LT-I	LT-I	20,480	2.5	8,190	64,000	22.0	2,910
LT-IIa	LT-IIb	4,000	1.0	4,000	8,000	1.7	4,700
LT-IIb	LT-IIa	9,000	6.4	1,400	128,000	22.2	5,820
LT-I	LT-IIa	640	—[c]	—	640	—	—
LT-I	LT-IIb	320	—	—	1,280	—	—
LT-IIa	LT-I	1,000	1.0	1,000	640	0.2	2,600
LT-IIb	LT-I	640	2.1	302	1,280	2.5	512

[a] One unit of toxin is the smallest dose needed to produce a response in 75 to 100% of cells in a Y1 adrenal cell bioassay (29). Cells expressing A subunit alone or B subunit alone were not toxigenic.

[b] The amount of holotoxin in each extract was measured by solid-phase radioimmunoassay (11). Extracts were incubated in microtiter wells sensitized with 20 μg each of GM1, GD1a, and GD1b, and probed with A subunit-specific MAb 4B6 (LT-I) (2), MAb 5C5 (LT-IIa), or MAb 19G5 (LT-IIb). In each case, the immunoreactivity was expressed as the amount of holotoxin containing the same A subunit that produces an equivalent response in the radioimmunoassay.

[c] —, MAb specific to the A subunit failed to bind to holotoxin.

trol extracts from strains expressing only A or B polypeptides were not cytotoxic.

RESULTS

To facilitate complementation studies, DNA fragments that encode individual A or B polypeptides of LTp-I, LT-IIa, or LT-IIb were isolated and cloned into compatible plasmid vectors (Table 1). Each A gene with its native promoter was cloned into pACYC184 (Clr^r, P15A replicon), and each B gene without its native promoter was cloned into pBluescript KS+ (Amp^r, ColE1 replicon) under control of the IPTG-inducible *lac* promoter. Pairs of the compatible plasmids carrying each possible combination of cloned A and B genes were transformed into *E. coli* XL1-Blue, and cotransformants were selected and maintained by growth in the presence of ampicillin and chloramphenicol. To control for artifacts resulting from different plasmid copy numbers or expression of the A and B genes from promoters of different strength, hybrid operons containing each combination of A and B genes expressed under control of the *lac* promoter in pBluescript KS+ were also constructed, introduced into *E. coli* XL1-Blue, and maintained under ampicillin selection.

Strains expressing each pair of A and B polypeptides, either on separate plasmids or as hybrid operons, were cultured in LB broth until mid-log phase and induced overnight with IPTG. Periplasmic contents were released by treatment with polymyxin B, and the extracts were tested for toxicity in Y1 adrenal cell assays and for immunoreactive holotoxin in solid-phase radioimmunoassays (Table 2). Extracts from negative controls that contained only an A or a B polypeptide were not toxic and contained no immunoreactive holotoxin (data not shown). Extracts from positive controls that contained the A and B polypeptides of a single enterotoxin (either LTp-I, LT-IIa, or LT-IIb) were highly toxic and contained substantial amounts of immunoreactive holotoxin with high specific activity. These positive controls demonstrated that LTp-I, LT-IIa, and LT-IIb can all be produced as mature periplasmic holotoxins in *E. coli* by complementation between the cloned genes for their A and B polypeptides.

Extracts from all of the strains that expressed genes for heterologous A and B polypeptides, including those that produced a combination of type I and type II polypeptides, were also toxic for Y1 adrenal cells (Table 2). The total toxic activity of extracts containing the hybrid toxins was usually less than the toxicity of extracts containing the wild-type holotoxins. Immunoreactive hybrid holotoxins were detected in all extracts, except those containing the A polypeptide of LTp-I and the B polypeptide of either LT-IIa or LT-IIb. The hybrid holotoxins in these exceptional extracts were also undetectable as antigens when any of several MAb against the A subunit of LTh-I (1, 2) was substituted for MAb 4B6 in the solid-phase radioimmunoassay (data not shown). Possible explanations for failure to detect these specific hybrid toxins in ganglioside-dependent solid-phase radioimmunoassays include failure of the A polypeptide to fold in a manner that permits expression of some conformation-dependent epitopes, masking of some epitopes of the A polypeptide by abnormal folding or by association with the heterologous B polypeptide, or deficient ganglioside-binding activity of the hybrid holotoxin. The amounts of toxic activity and immunoreactive holotoxin produced by strains that expressed a hybrid enterotoxin operon under control of the *lac* promoter in pBluescript KS+ were usually greater than the amounts produced by strains that expressed the same A and B polypeptides from separate, compatible plasmids.

To confirm by an independent method that toxicity was due to formation of hybrid holotoxins, extracts containing each combination of A and B polypeptides were adsorbed with Sepharose–streptococcal G-protein beads coated with MAb against the B polypeptides that they contained (Table 3). None of the MAb against a specific B polypeptide cross-reacted with any of the A polypeptides or with the other B polypeptides (data not shown). Purified LTp-I, LT-IIa and LT-IIb toxins were used as positive controls, and Sepharose–streptococcal G-protein beads coated with MAb specific for the other B polypeptides served as negative controls for the absorption experiments. In each case, toxicity was removed by immunoabsorption only when the

TABLE 3. Removal of biological activity of heat-labile enterotoxins by adsorption with B subunit-specific MAb

Toxin Composition[a]		B subunit-specific MAb[b]	Biological activity removed (%)
Subunit A	Subunit B		
LT-IIa	LT-IIa	15G3	>88
LT-IIb	LT-IIb	20C9	>94
LT-I	LT-I	12G5	99
LT-IIa	LT-IIb	20C9	94
LT-IIb	LT-IIa	15G3	>99
LT-I	LT-IIa	15G3	88
LT-I	LT-IIb	20C9	0[c]
LT-IIa	LT-I	12G5	94
LT-IIb	LT-I	12G5	94
LT-IIa[d]	LT-IIa[d]	15G3	99
LT-IIb[d]	LT-IIb[d]	20C9	88
LT-I[d]	LT-I	12G5	97

[a] Toxin-containing bacterial extracts (unless otherwise noted) were prepared from strains expressing toxin from wild-type or hybrid operons.
[b] Specificities of MAb used: 15G3 (LT-IIa B subunit), 20C9 (LT-IIb B subunit), and 12G5 (LT-I B subunit) (2). The antibodies do not cross-react.
[c] MAb 20C9 does not bind the hybrid holotoxin.
[d] Purified holotoxins were used as positive controls.

Sepharose–streptococcal G-protein beads were coated with MAb against the B polypeptide that was present in the extract or purified enterotoxin. The only exception was the extract that contained the A polypeptide of LTp-I and the B polypeptide of LT-IIb, from which toxicity was not absorbed by using MAb 20C9 or an alternative MAb 11E5 that was also specific for the B polypeptide of LT-IIb (data not shown). The hybrid holotoxin that failed to react with MAb against the B polypeptides in the immunoabsorption experiments also failed to react with MAb against the A polypeptide in the solid-phase radioimmunoassays shown in Table 2. These results were confirmed in several experiments using independently derived constructs for expression of the LTp-I A polypeptide and the LT-IIb B polypeptide.

Previous studies reported that GM1 is the preferred ganglioside receptor for LT-I (13) and that LT-IIa and LT-IIb preferentially bind to the gangliosides GD1b and GD1a, respectively (11). To confirm that the B polypeptides determine ganglioside-binding specificity for both type I and type II heat-labile enterotoxins, extracts from strains used for the complementation tests were tested in solid-phase radioimmunoassays to determine the relative binding activities of the corresponding hybrid holotoxins for GM1, GD1a, and GD1b (Fig. 1). In each case, the B polypeptide of the hybrid holotoxin determined its ganglioside-binding specificity. Hybrid holotoxins containing A polypeptides of LTp-I and B polypeptides of LT-IIa or LT-IIb were not tested, because we were unable to detect them in ganglioside-binding solid-phase radioimmunoassays with anti-A MAb.

Finally, the ability of various combinations of A and B polypeptides to form holotoxins in vitro was examined (Table 4). Previous studies demonstrated that mixtures of denatured A and B polypeptides of type I enterotoxins in urea can renature into active holotoxin when the urea is slowly removed by dialysis (16, 44), but holotoxin is not formed when the A and B subunits are simply mixed together in aqueous solutions at neutral pH (44). We confirmed these findings for mixtures of A and B polypeptides from LTp-I. Unexpectedly, we found that toxic, immunoreactive holotoxins formed spontaneously in mixtures of extracts containing any combination of A and B polypeptides of LT-IIa and LT-IIb, without subjecting them to denaturation in urea and dialysis. Furthermore, type II holotoxins also formed when extracts containing A polypeptides were added to ganglioside-coated wells to which B polypeptides had previously been adsorbed. Hybrid holotoxins were not formed from any mixture that combined a type I A polypeptide with a type II B polypeptide, or vice versa. These data demonstrated that the requirements for assembly of type II enterotoxins differ significantly from those required for assembly of type I toxins.

DISCUSSION

The assembly of multimeric proteins such as heat-labile enterotoxins requires the interaction of complementary domains of the constituent subunits and is dependent on subunit conformation (34). Recently, the three-dimensional structure of LTp-I was solved by X-ray diffraction (43). The A2 domain of LTp-I is inserted into a pore formed at the center of the B pentamer, and the A2 domain interacts noncovalently with alpha helical domains of the B polypeptides to form holotoxin. Since LT-I and CT are highly homologous in these regions of their A and B polypeptides, it is not surprising that hybrid holotoxins composed of purified LT-I and CT subunits can form stable and biologically active molecules. Experiments presented in this paper demonstrate that hybrid holotoxins comprised of type I and II subunits also can be formed, although the A2 domains of type I and II enterotoxins show only moderate amino acid sequence homology and the B polypeptides of type I and II enterotoxins have little, if any, significant homology. The data suggest that type I and type II toxins have common structural features that are required for holotoxin assembly but are not readily apparent from their primary amino acid sequences.

Hybrid toxins were expressed from strains containing all

TABLE 4. In vitro formation of biologically active holotoxin in mixtures of extracts containing A and B subunits

Toxin composition[a]		Immunoreactivity (μg/ml)[b]	Cytotoxicity[c]
Subunit A	Subunit B		
LT-IIa		0	−
LT-IIb		0	−
LT-I		0	−
	LT-I	0	−
	LT-IIa	0	−
	LT-IIb	0	−
LT-IIa	LT-IIa	2.0	+
LT-IIb	LT-IIb	1.9	+
LT-IIa	LT-IIb	2.8	+
LT-IIb	LT-IIa	12.5	+
LT-I	LT-IIa	0	−
LT-I	LT-IIb	0	−
LT-IIa	LT-I	0	−
LT-IIb	LT-I	0	−

[a] Equal volumes (10 to 50 μl) of polymyxin B sulfate extracts from strains expressing LT-II A or B subunits were mixed and incubated for 10 min at 37°C prior to the solid-phase radioimmunoassay assay for holotoxin or the Y1 adrenal cell assay for cytotoxicity (29).
[b] Specificities of the MAb used in the solid-phase radioimmunoassay: MAb 4B6 (LT-I) (2), MAb 5C5 (LT-IIa), and MAb 19G5 (LT-IIb).
[c] The mixture caused either a +4 rounding response (+) or no rounding (−) in a Y1 adrenal cell cytotoxicity assay.

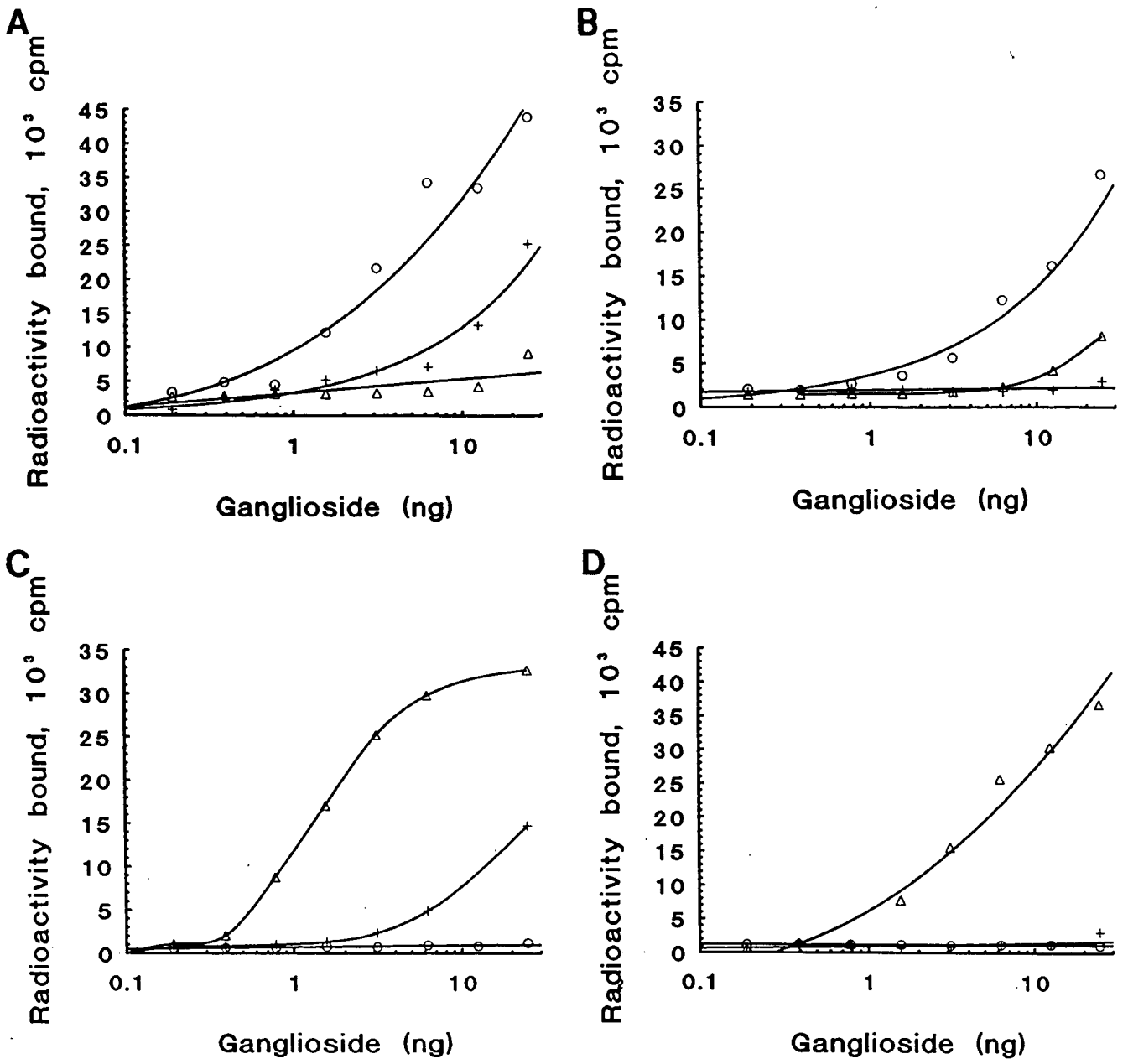
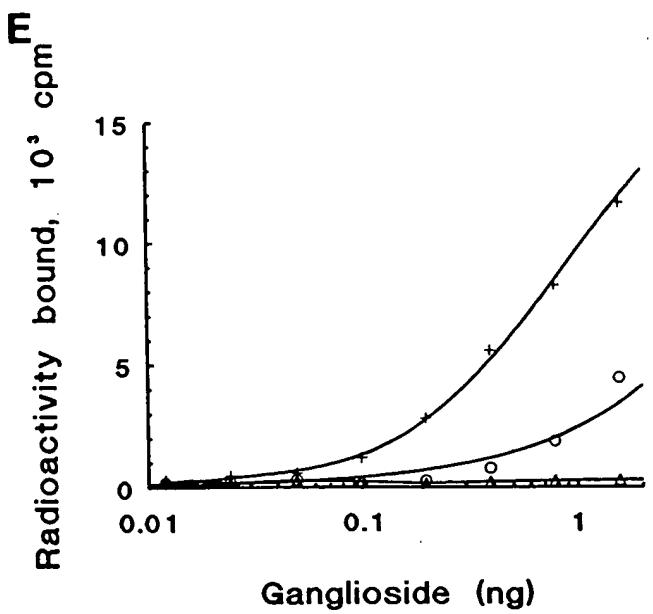
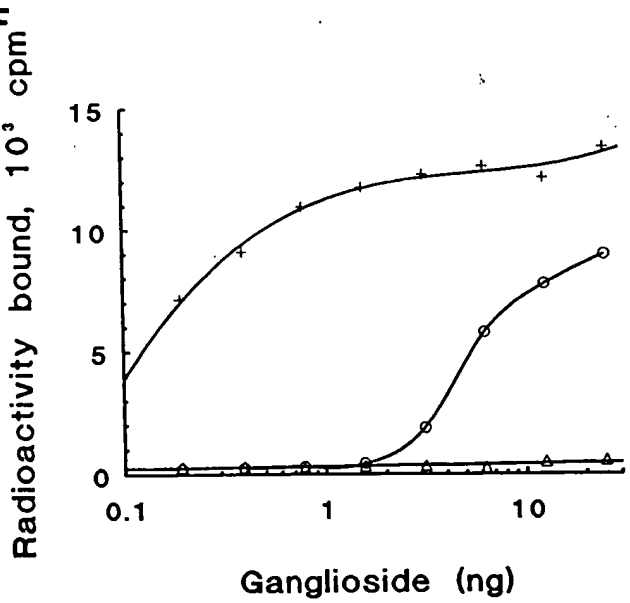


FIG. 1. Relative ganglioside-binding activities of toxins formed from homologous and heterologous combinations of A and B subunits from *E. coli* type I and type II enterotoxins. (A) Both A and B polypeptides from LT-IIa; (B) A polypeptide from LT-IIb and B polypeptide from LT-IIa; (C) both A and B polypeptides from LT-IIb; (D) A polypeptide from LT-IIa and B polypeptide from LT-IIb; (E) both A and B polypeptides from LTp-I; (F) A polypeptide from LT-IIa and B polypeptide from LTp-I; (G) A polypeptide from LT-IIb and B polypeptide from LTp-I. +, GM1; △, GD1a; ○, GD1b.

pairwise combinations of A and B genes from type I and type II enterotoxins on complementary plasmids or in operon fusions. The hybrid enterotoxins were usually detected in smaller amounts than the wild-type toxins containing A and B subunits, both in strains with complementing plasmids and in strains expressing both toxin subunits from a single operon fusion. Previous investigations have shown that prior treatment with trypsin was necessary to obtain the fully nicked form of purified LT-IIb toxin and to demonstrate its maximal toxicity (15). Attempts by Western blotting (immunoblotting) to determine the degree of nicking in these hybrid toxins were inconclusive, and treatment of the crude extracts with trypsin did not increase their toxicity (data not shown).

Extracts containing LTp-I A polypeptides and either LT-IIa or LT-IIb B polypeptides were slightly toxic on Y1 cells, but immunoreactive toxin was not demonstrated with any MAb among several that were specific for the A subunit of LTp-I or the B subunit of LT-IIa or LT-IIb. It is possible that specific epitopes on these particular hybrid toxins are conformationally altered or obscured. Alternatively, these particular hybrid toxins may be highly toxic but expressed at levels that are below the limit of sensitivity of the solid-phase





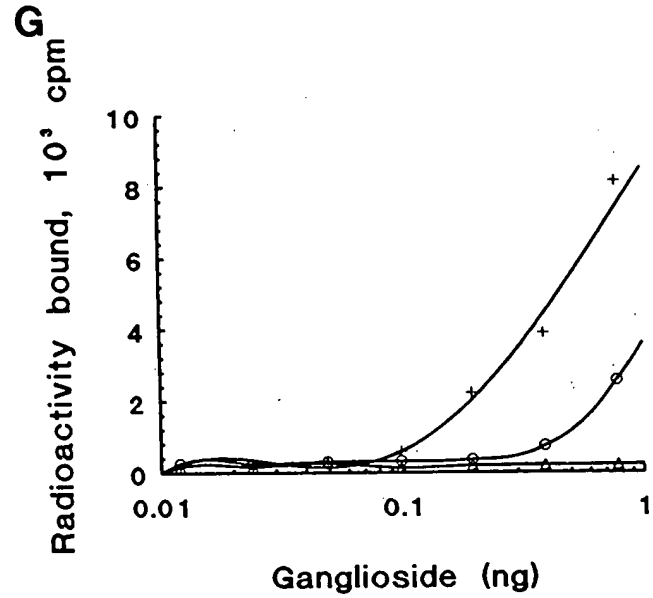


FIG. 1—*Continued.*

radioimmunoassays used in our experiments (approximately 100 pg per assay).

To demonstrate that the B polypeptides of type II enterotoxins determine their ganglioside-binding activity and to investigate the effects, if any, of different A polypeptides in modifying the ganglioside-binding activity or specificity of the B polypeptides in hybrid holotoxins, we studied the ganglioside-binding activities of all of the wild-type and hybrid holotoxins that we prepared. Our results demonstrated that each of the hybrid toxins maintained the same preference for ganglioside-binding that was exhibited by the native holotoxin with the same B subunit. We conclude that the B subunits alone determine ganglioside-binding specificities for both LT-I and LT-II enterotoxins.

Assembly of type I enterotoxins is thought to occur in a stepwise manner in vivo. The A polypeptides of type I enterotoxins do not assemble with mature B pentamers either in vivo or in vitro; instead, they appear to interact with an intermediate complex of B polypeptides (B_n, where n is less than 5) (18). Assembly of type I holotoxins does occur in vitro when A polypeptides and monomeric B polypeptides are denatured in acidic urea and slowly renatured by dialysis against appropriate buffers (16, 44). In contrast, the experiments reported here demonstrated that either homologous or heterologous mixtures of A and B polypeptides of LT-IIa and LT-IIb assembled readily in vitro, without prior exposure to denaturing conditions, to form immunoreactive and biologically active holotoxins. Further experiments will be needed to investigate the kinetics and stoichiometry for assembly of purified subunits of type II enterotoxins in vitro and compare the pathways for assembly of type II enterotoxins in vivo and in vitro.

In summary, all homologous and heterologous combinations of A and B polypeptides from type I and type II heat-labile enterotoxins are capable of assembling into active holotoxins in vivo, although not with equal efficiency. The data suggest that certain structural features necessary for interaction of A and B polypeptides during holotoxin assembly have been conserved among type I and type II enterotoxins, but these features are not evident from primary amino acid sequences. Determination of the three-dimensional structure of a representative type II enterotoxin is an important goal and will permit direct comparisons of the interactions between the A and B polypeptides in mature type I and type II heat-labile enterotoxins.

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US00603[illegible]4A

United States Patent [19]

Russell et al.

[11] **Patent Number: 6,030,624**

[45] **Date of Patent: Feb. 29, 2000**

[54] **MUCOSAL IMMUNOGENS FOR NOVEL VACCINES**

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[73] Assignee: **UAB Research Foundation**, Birmingham, Ala.

[21] Appl. No.: **08/912,180**

[22] Filed: **Aug. 15, 1997**

Related U.S. Application Data

[60] Provisional application No. 60/024,074, Aug. 16, 1996, abandoned.

[51] **Int. Cl.**[7] **A61K 39/02**; A61K 39/09; C12N 1/21

[52] **U.S. Cl.** **424/200.1**; 424/93.2; 424/244.1; 435/252.3; 435/252.8

[58] **Field of Search** 435/320.1, 252.3, 435/252.33, 252.8; 530/69.3, 350, 403; 424/192.1, 197.11, 200.1, 236.1, 244.1, 261.1, 93.2

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Hajishengallis et al. Journal of Immunology 154 (9): 4322–32, May 1, 1995.

Redman et al. Infection and Immunity 63 (5): 2004–2011, May 1995.

Primary Examiner—Mary E. Mosher

Attorney, Agent, or Firm—Benjamin Aaron Adler

[57] **ABSTRACT**

The present invention provides chimeric proteins such as Salivary Binding Protein (SBR) coupled to the B subunit of cholera toxin. Such a chimeric protein, when expressed in attenuated *Salmonella typhymurium* produces significant increases in serum IgG and salivary IgA antibody levels after oral immunization. In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin A2/B chimeric protein expressed in *E. coli*. Intragastric immunization of SBR coupled to CTB in this chimeric protein form leads to increased antigen responsive T cells. In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin$^{\Delta A1}$ chimeric protein expressed in *Salmonella typhimurium*. Oral immunization using this recombinant plasmid results in increased serum IgG responses to antigen. Oral immunization using this recombinant plasmid also resulted in increased salivary IgA antibody res[illegible]ses to antigen.

1[illegible] Claims, 26 Drawing Sheets

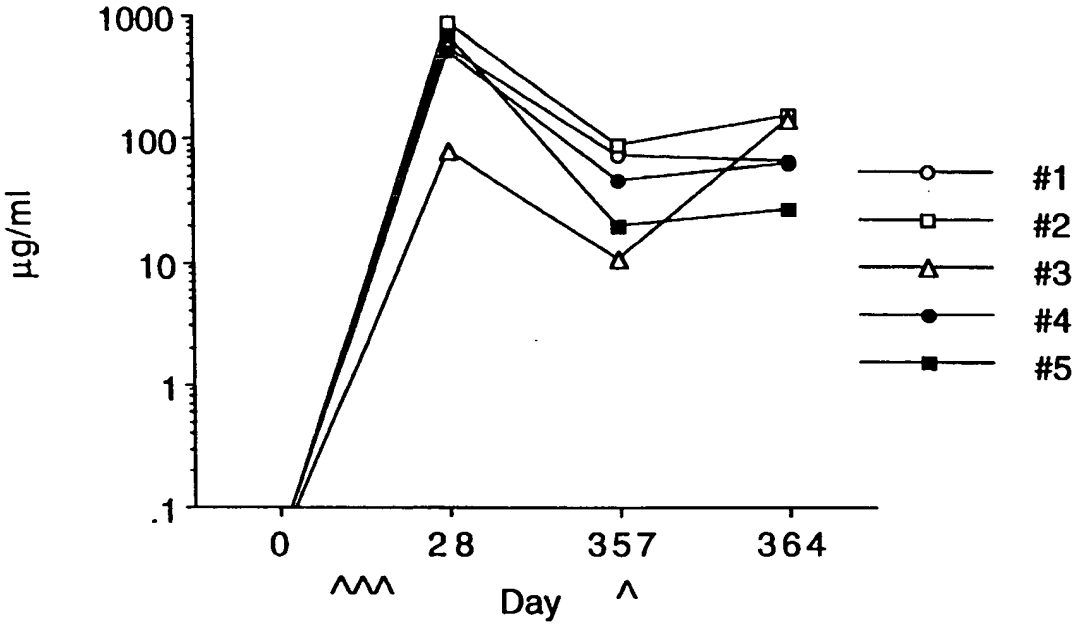
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 1A

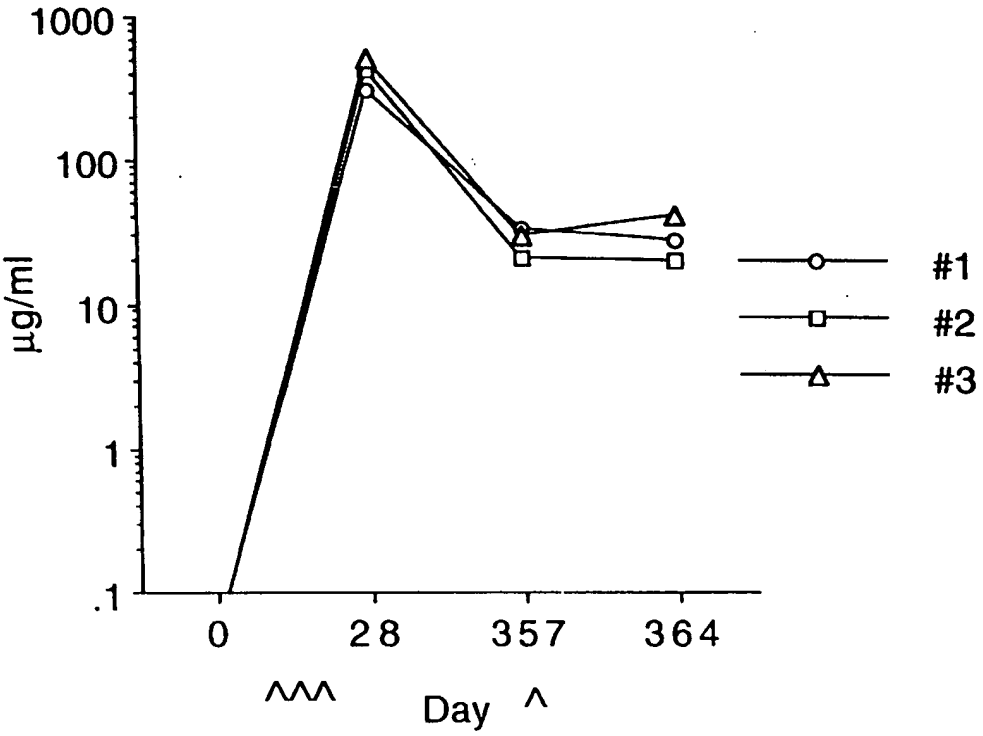
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3

Fig. 1B

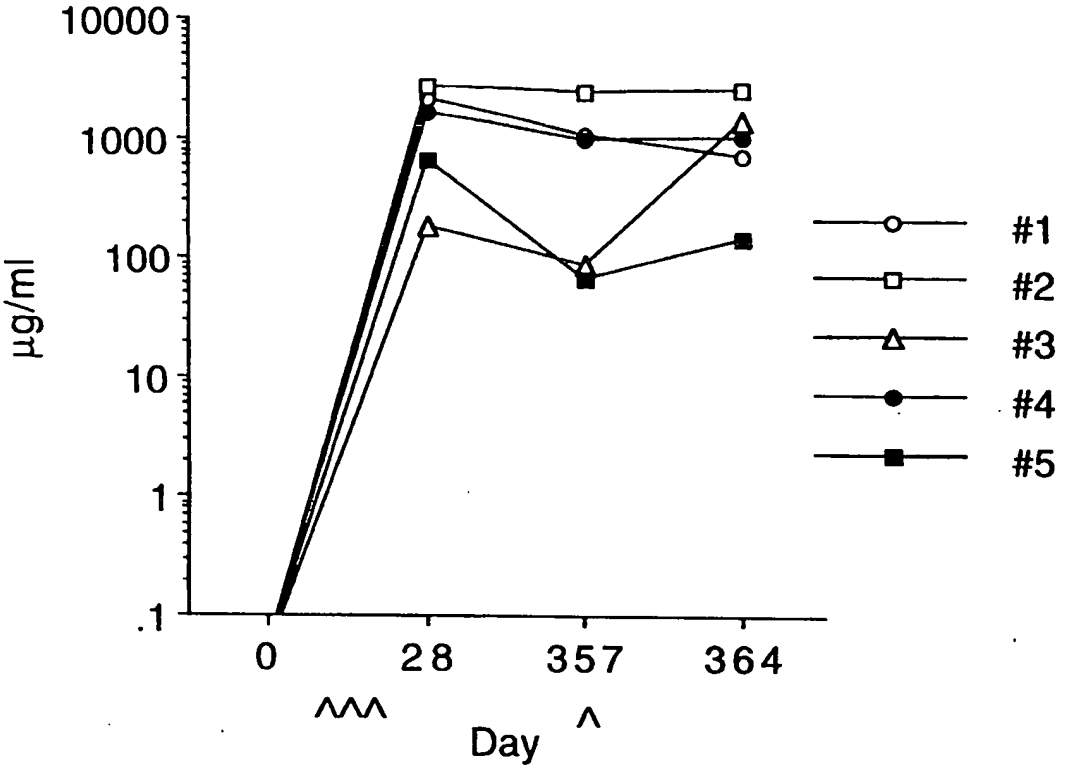
10000
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 1C

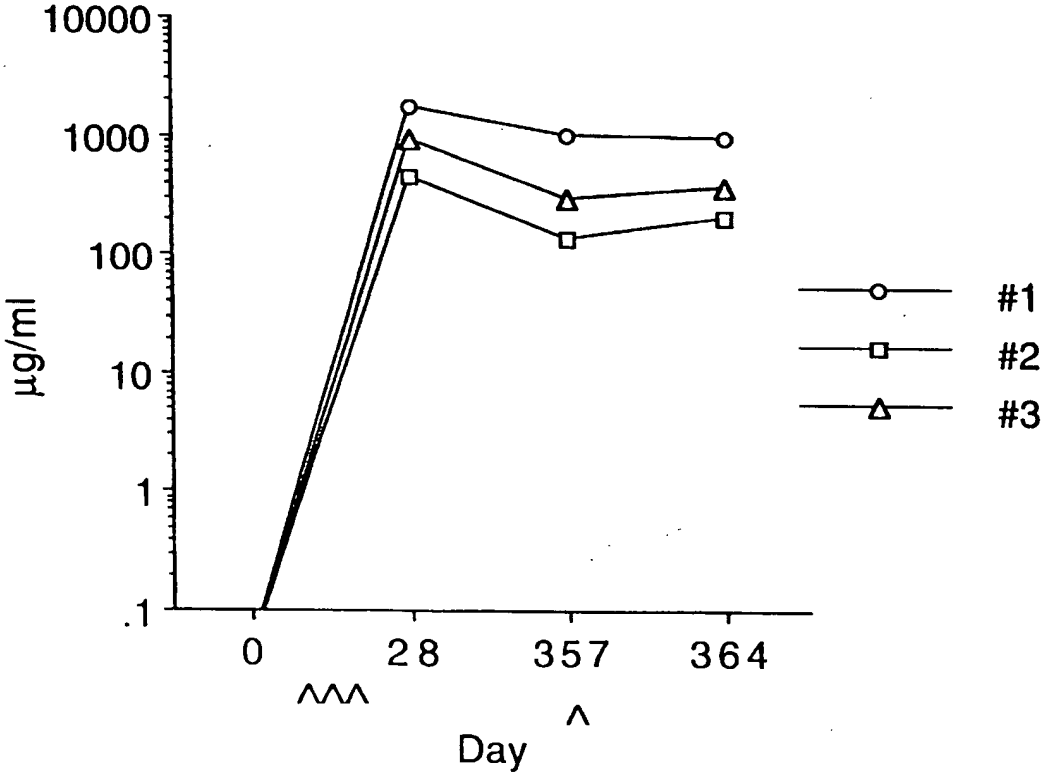
10000
1000
100
10
1
.1
µg/ml
0
28
357
364
Day
#1
#2
#3

Fig. 1D

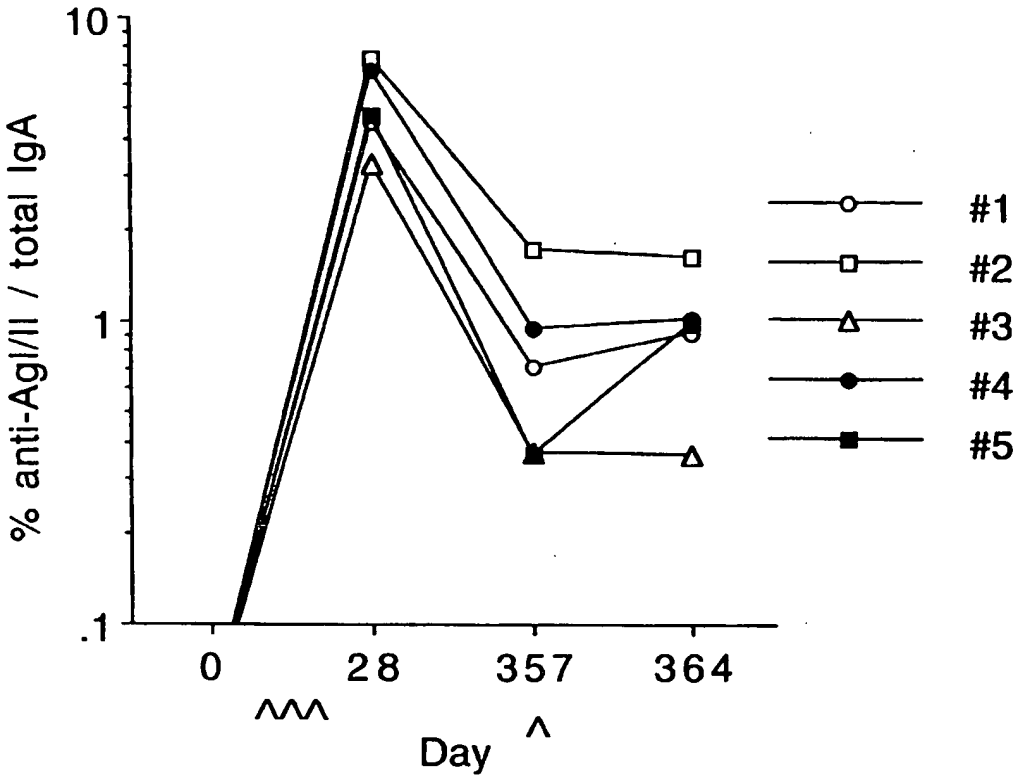
10
1
.1
% anti-AgI/II / total IgA
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 2A

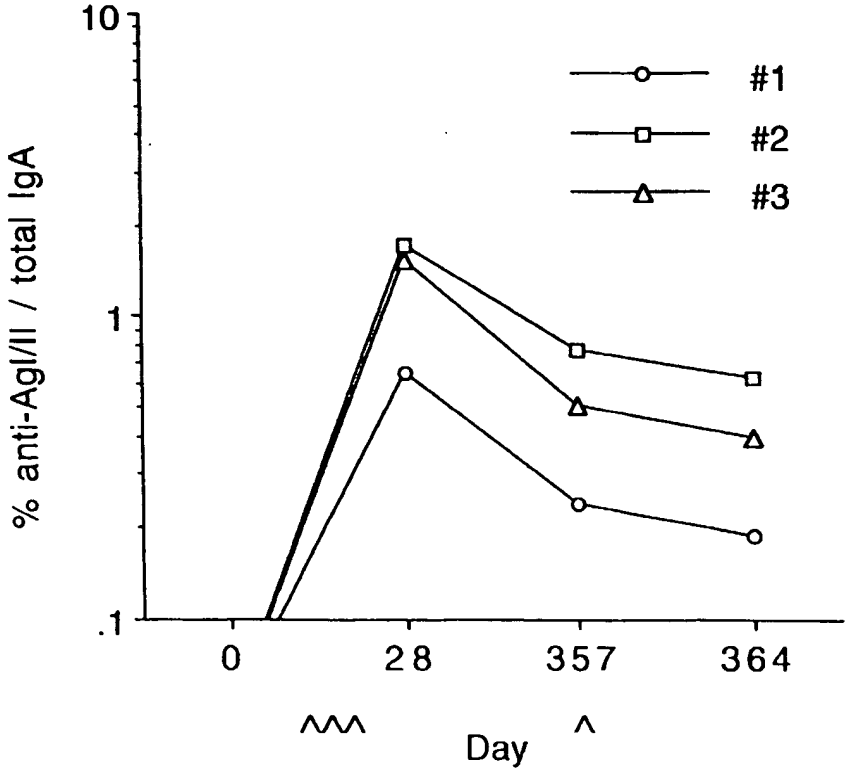
10
1
.1
% anti-AgI/II / total IgA
0
28
357
364
Day
#1
#2
#3

Fig. 2B

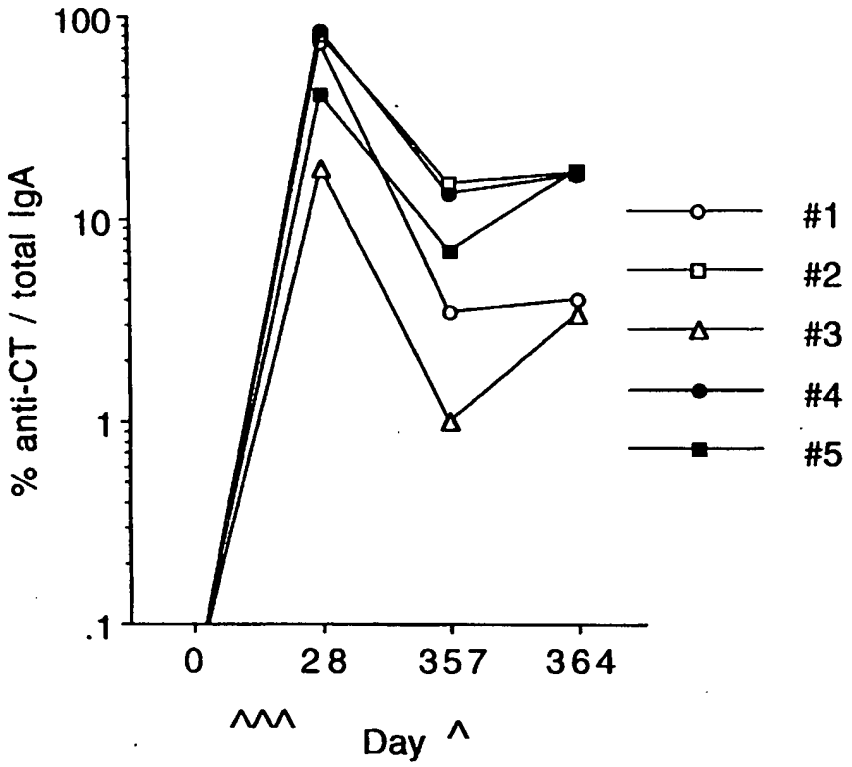
100
10
1
.1
% anti-CT / total IgA
0
28
357
364
Day
#1
#2
#3
#4
#5

Fig. 2C

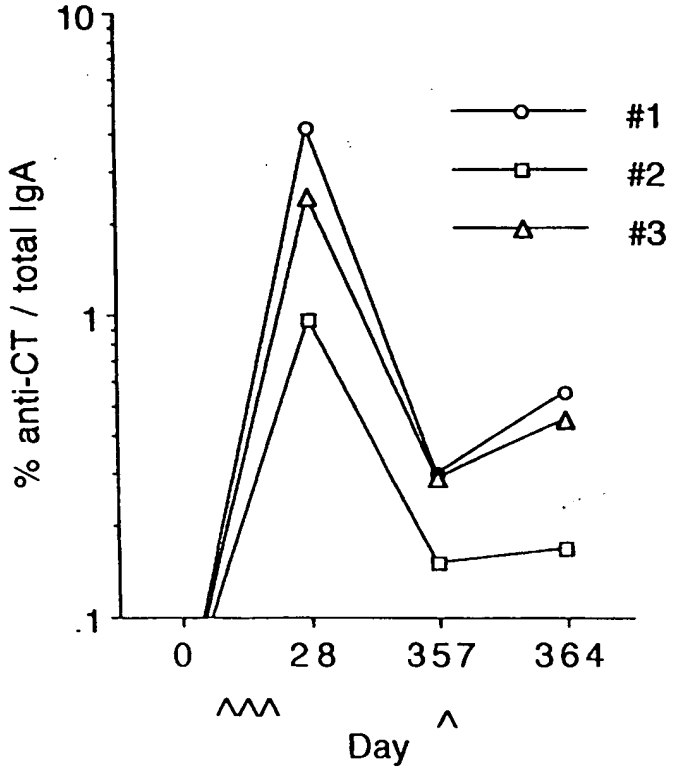
10
1
.1
% anti-CT / total IgA
0
28
357
364
Day
#1
#2
#3

Fig. 2D

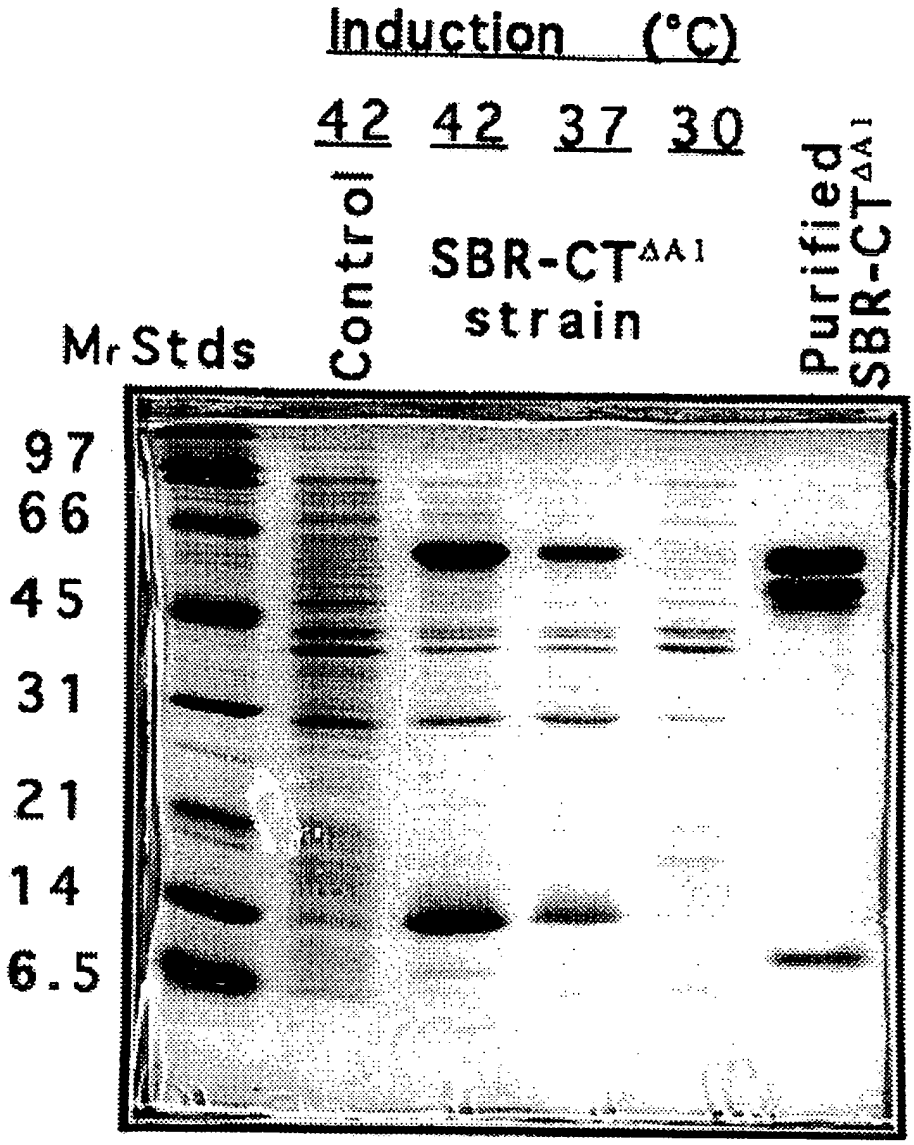
Induction (°C)
42
42
37
30
Control
SBR-CT$^{\Delta A1}$ strain
Purified SBR-CT$^{\Delta A1}$
Mr Stds
97
66
45
31
21
14
6.5

FIG. 3

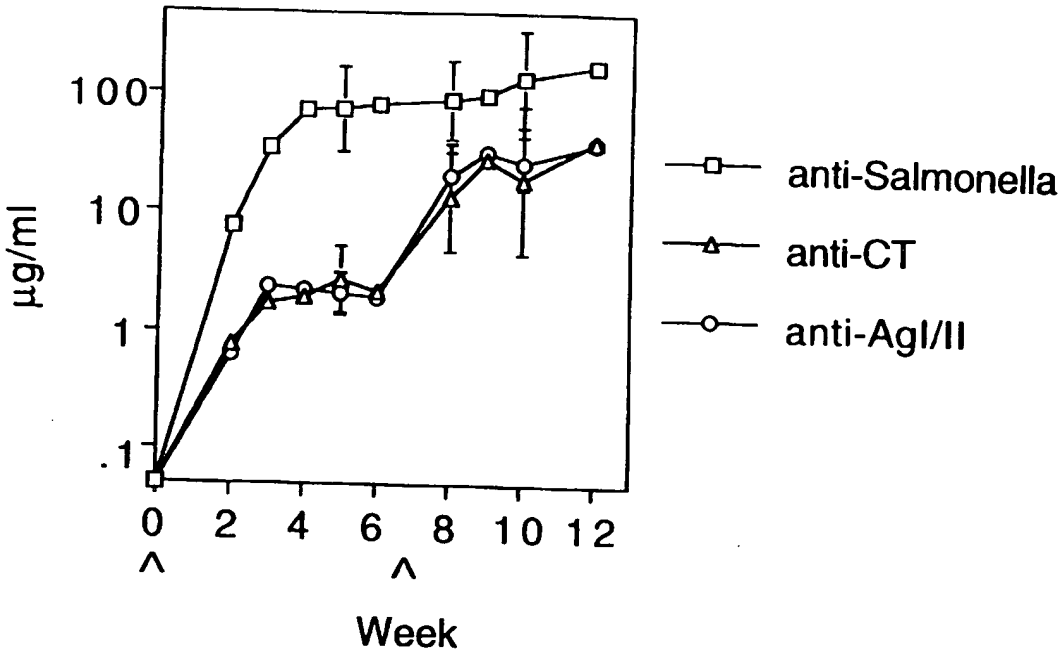
100
10
1
.1
µg/ml
0 2 4 6 8 10 12
Week
anti-Salmonella
anti-CT
anti-AgI/II

Fig. 4A

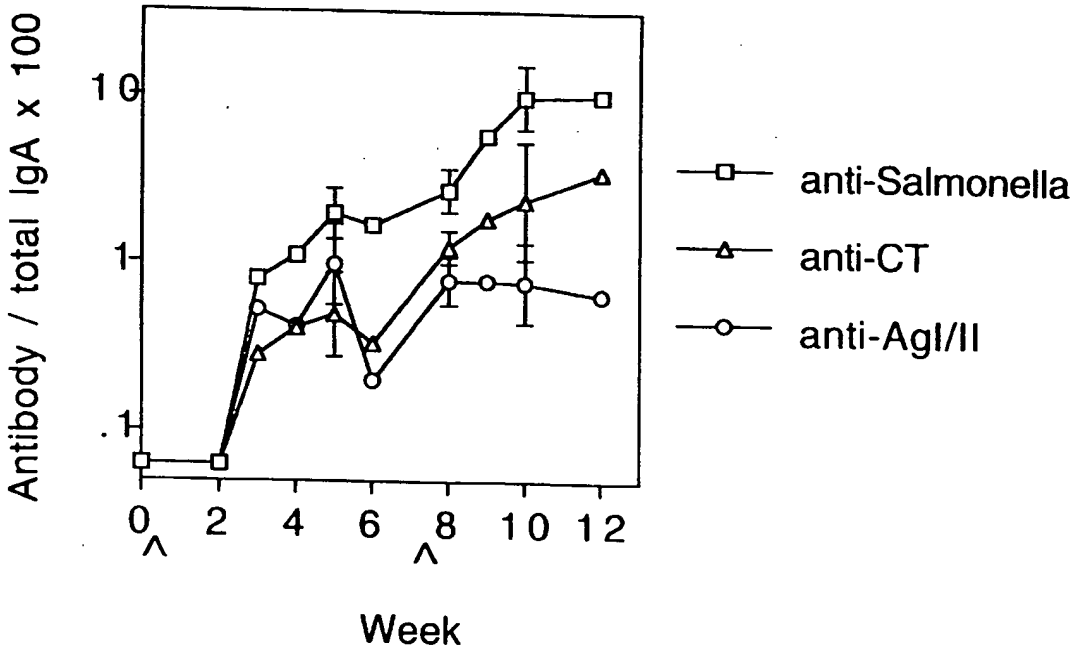
10
1
.1
Antibody / total IgA x 100
0 2 4 6 8 10 12
Week
anti-Salmonella
anti-CT
anti-AgI/II

Fig. 4B

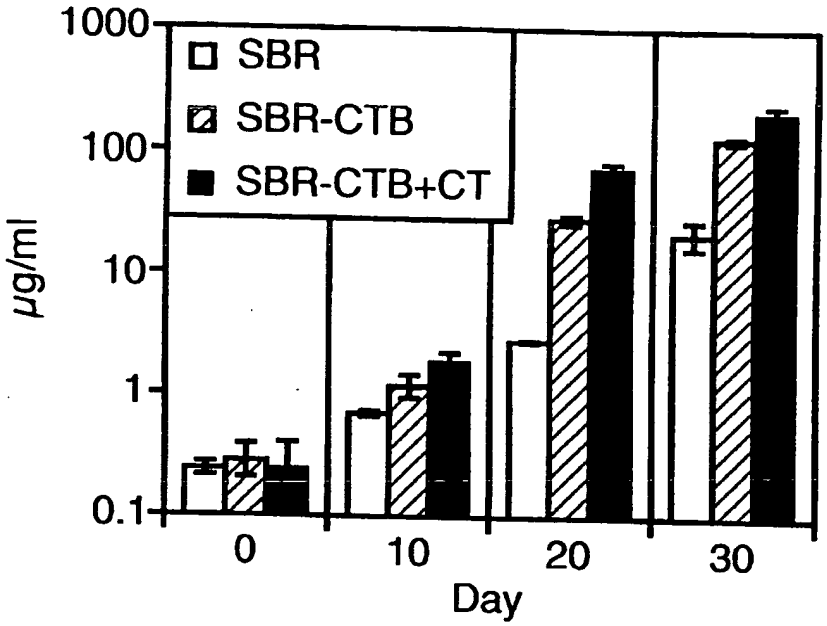
1000
100
10
1
0.1
μg/ml
SBR
SBR-CTB
SBR-CTB+CT
0
10
20
30
Day

Fig. 5A

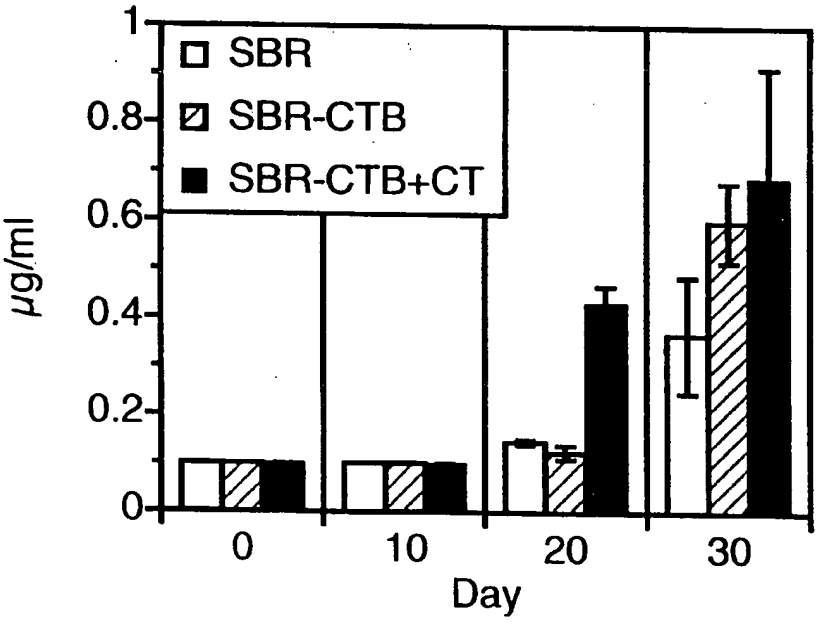
1
0.8
0.6
0.4
0.2
0
μg/ml
SBR
SBR-CTB
SBR-CTB+CT
0
10
20
30
Day

Fig. 5B

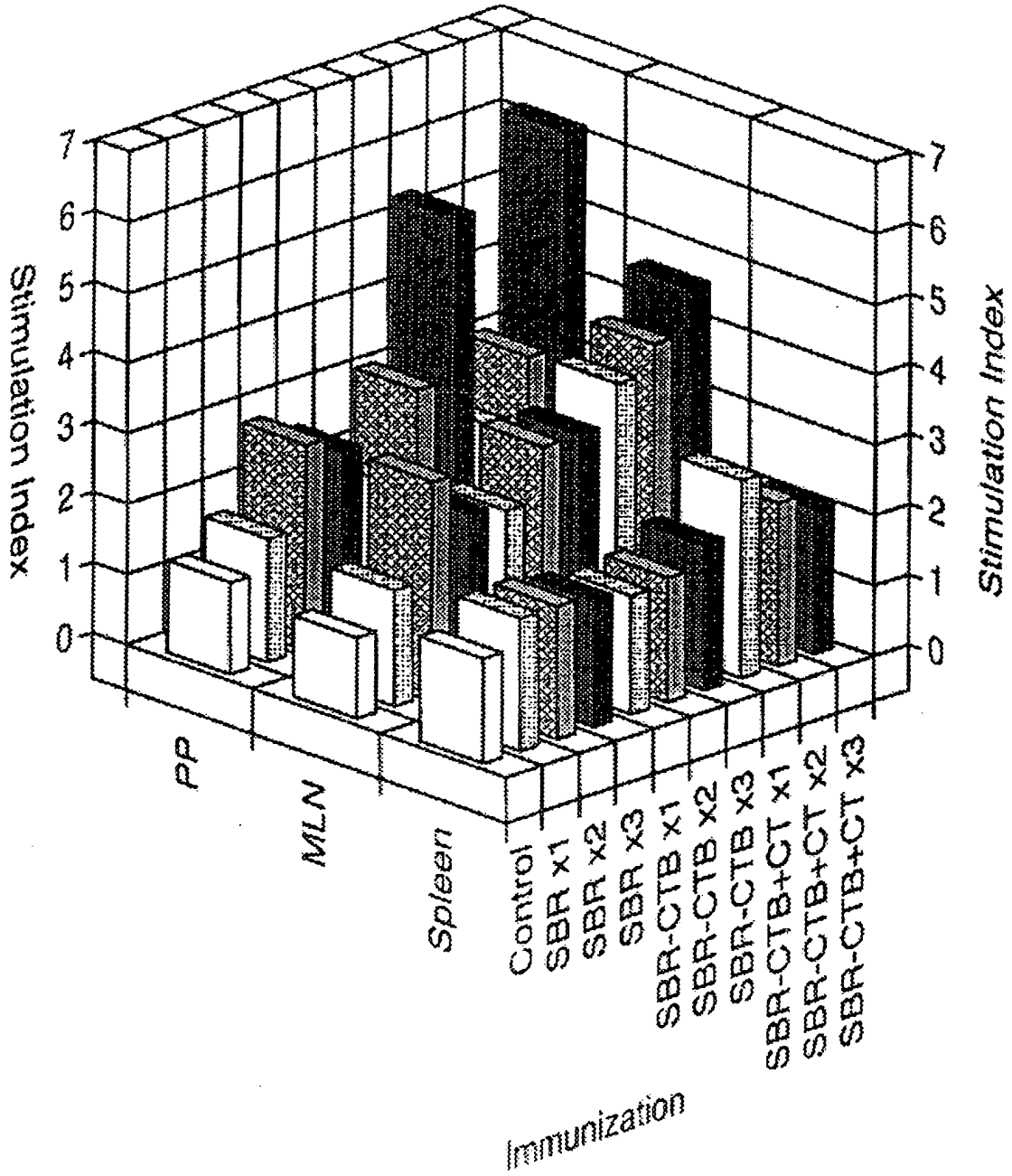
Stimulation Index
0
1
2
3
4
5
6
7
PP
MLN
Spleen
Control
SBR x1
SBR x2
SBR x3
SBR-CTB x1
SBR-CTB x2
SBR-CTB x3
SBR-CTB+CT x1
SBR-CTB+CT x2
SBR-CTB+CT x3
Immunization
Stimulation Index

Fig. 6

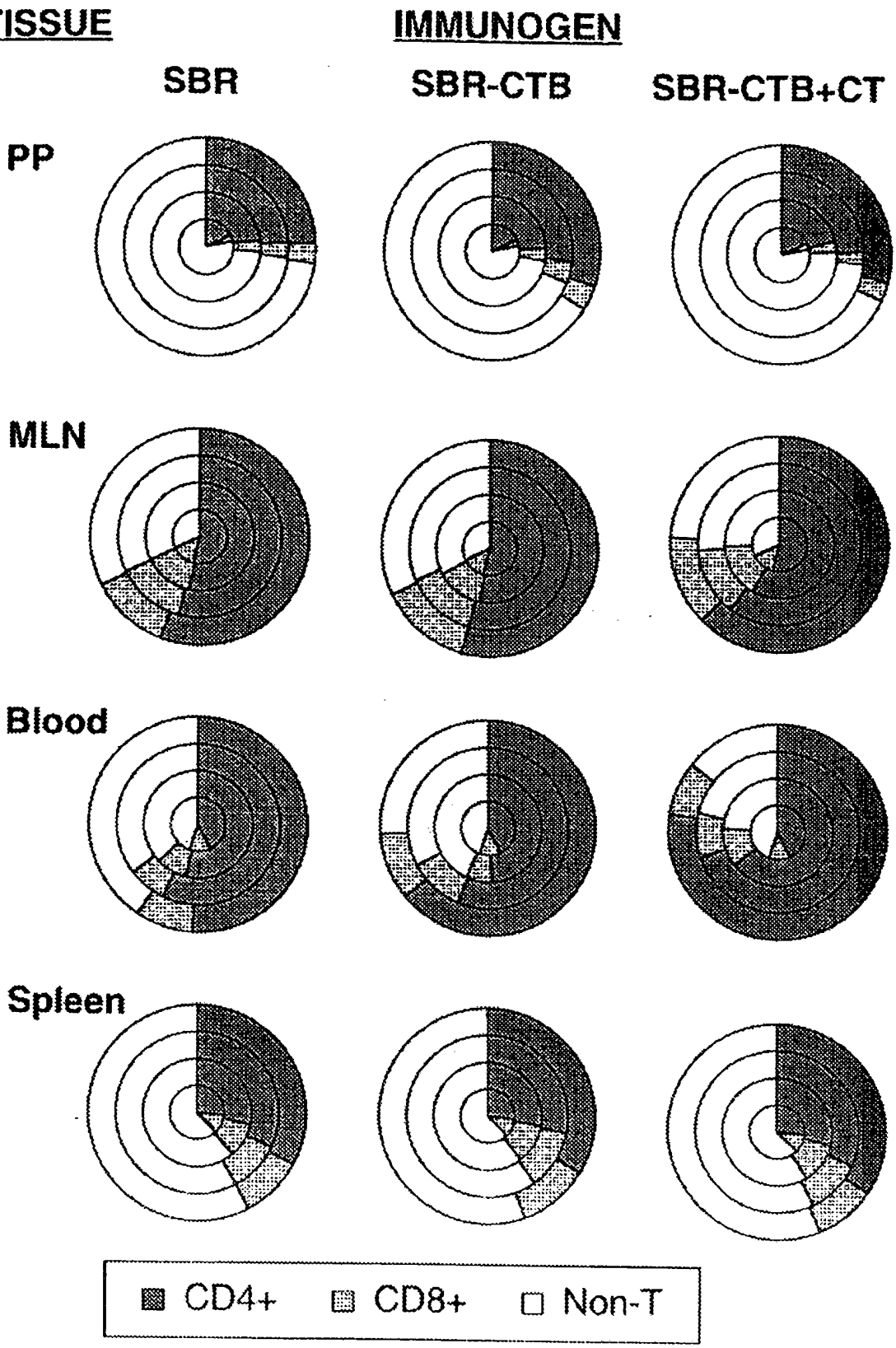
TISSUE
IMMUNOGEN
SBR
SBR-CTB
SBR-CTB+CT
PP
MLN
Blood
Spleen
CD4+
CD8+
Non-T

Fig. 7

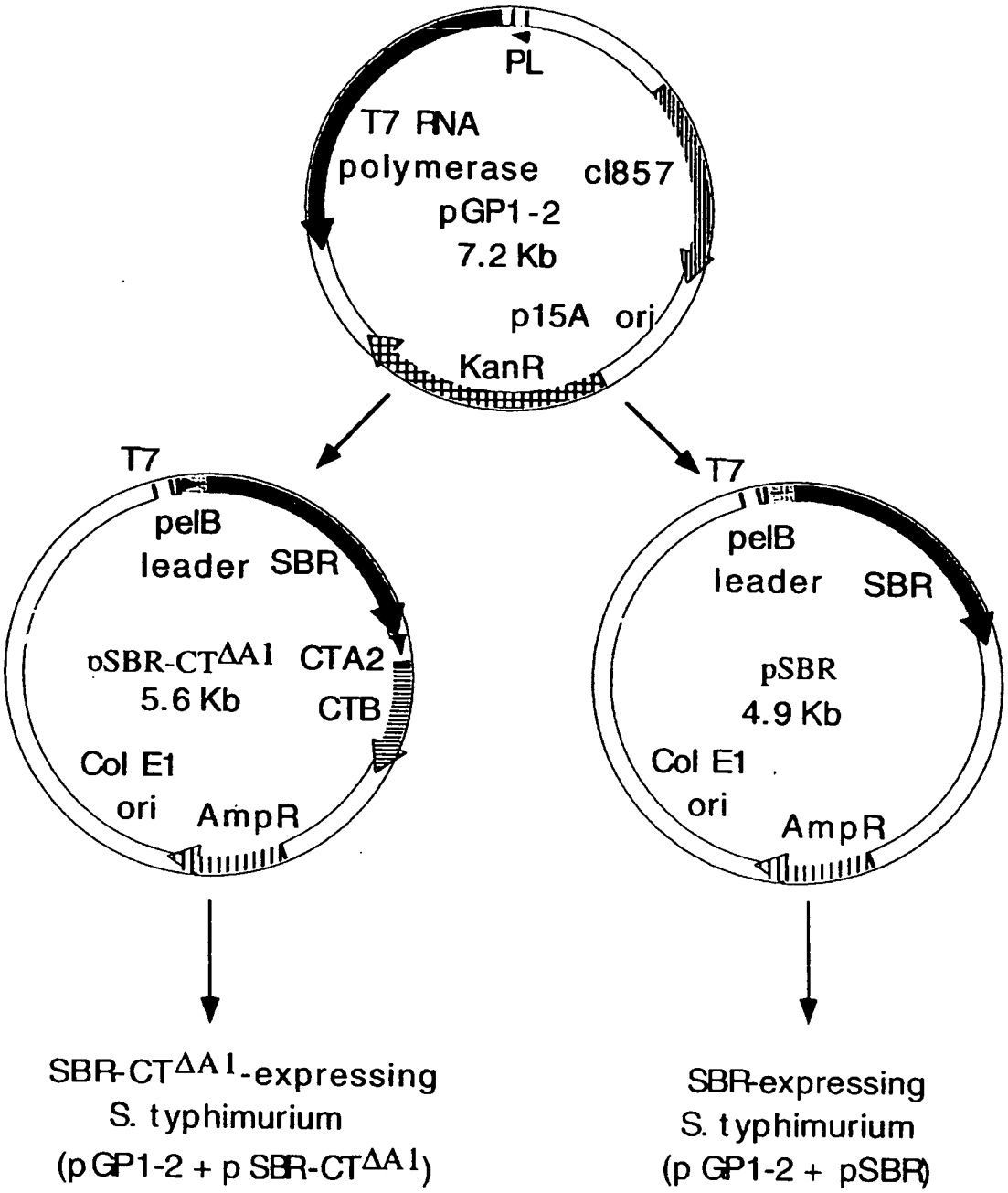
PL
T7 RNA
polymerase
cI857
pGP1-2
7.2 Kb
p15A ori
KanR
T7
pelB
leader
SBR
pSBR-CTΔA1
5.6 Kb
CTA2
CTB
Col E1
ori
AmpR
T7
pelB
leader
SBR
pSBR
4.9 Kb
Col E1
ori
AmpR
SBR-CTΔA1-expressing
S. typhimurium
(pGP1-2 + pSBR-CTΔA1)
SBR-expressing
S. typhimurium
(pGP1-2 + pSBR)

Fig. 8A

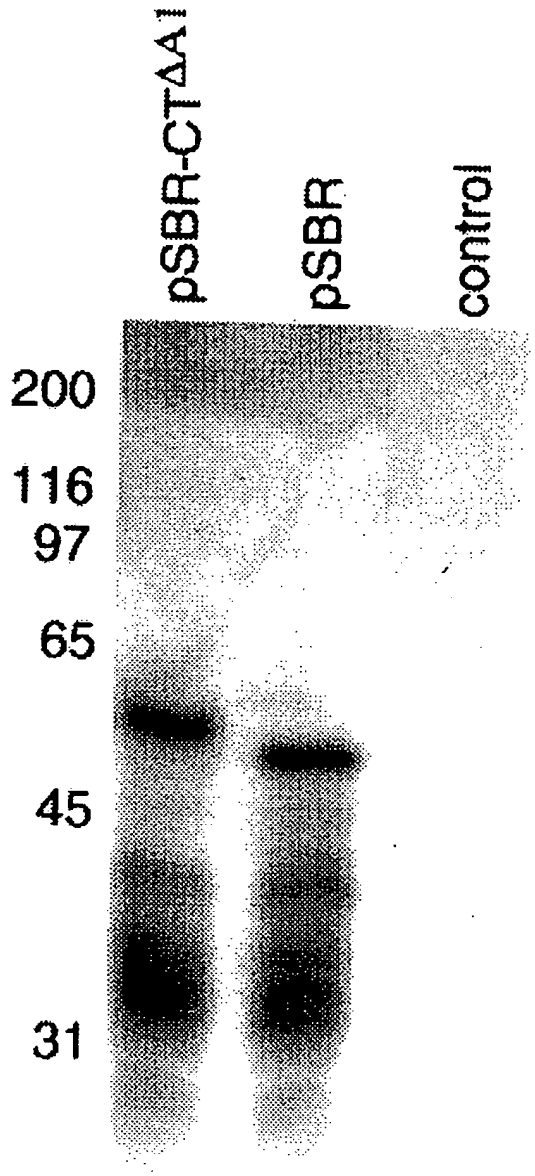

FIG. 8B

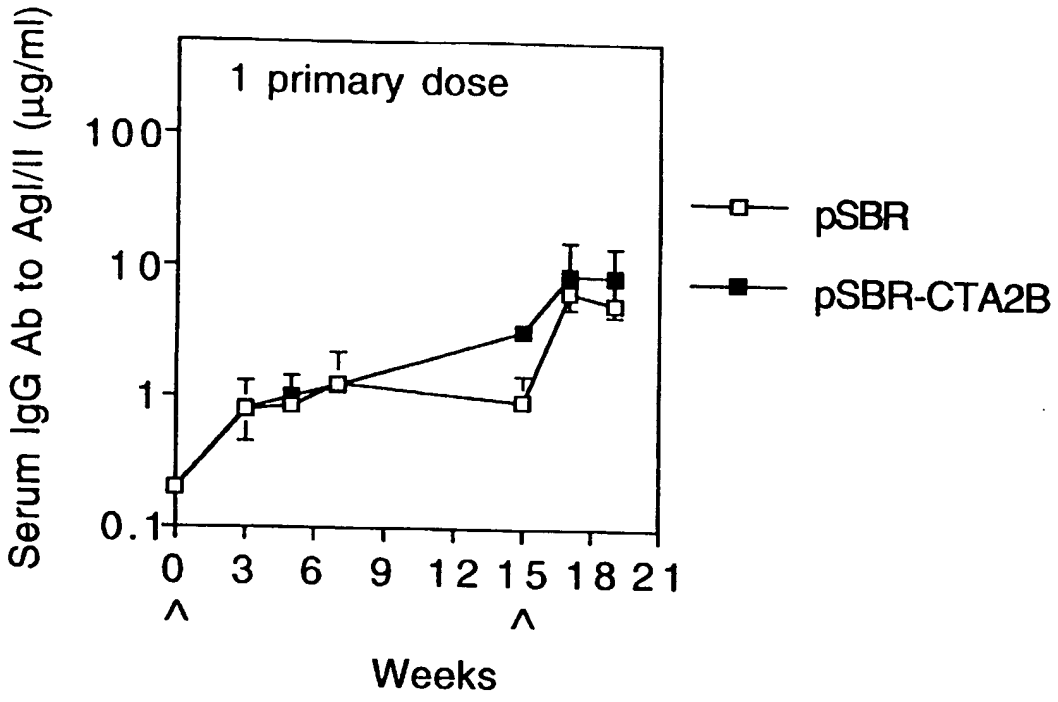
1 primary dose
Serum IgG Ab to AgI/II (µg/ml)
100
10
1
0.1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2B

Fig. 9A

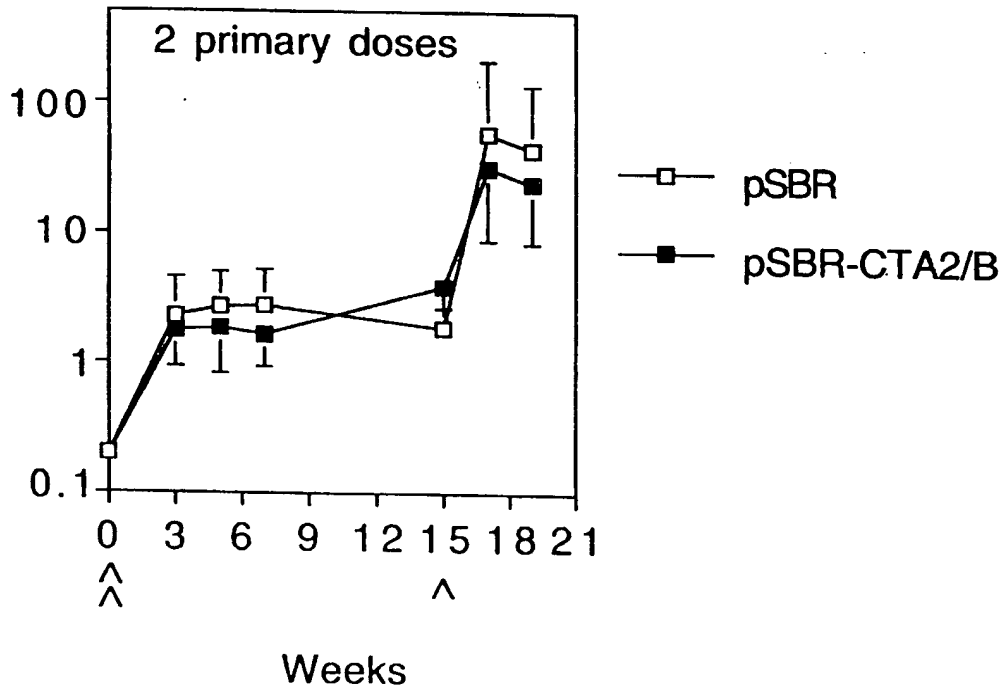
2 primary doses
100
10
1
0.1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2/B

Fig. 9B

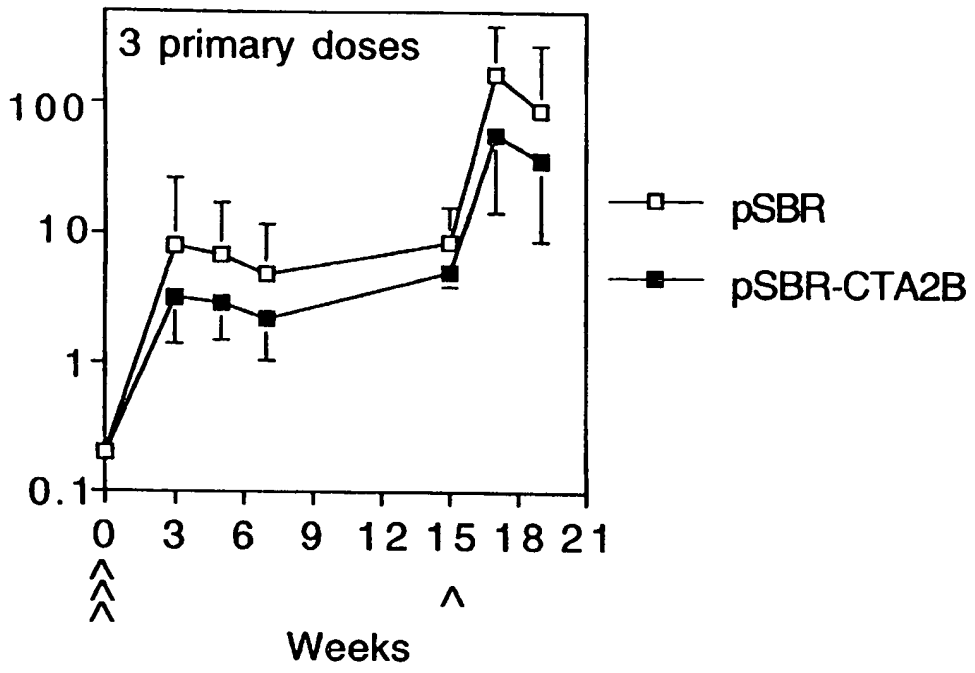

Fig. 9C

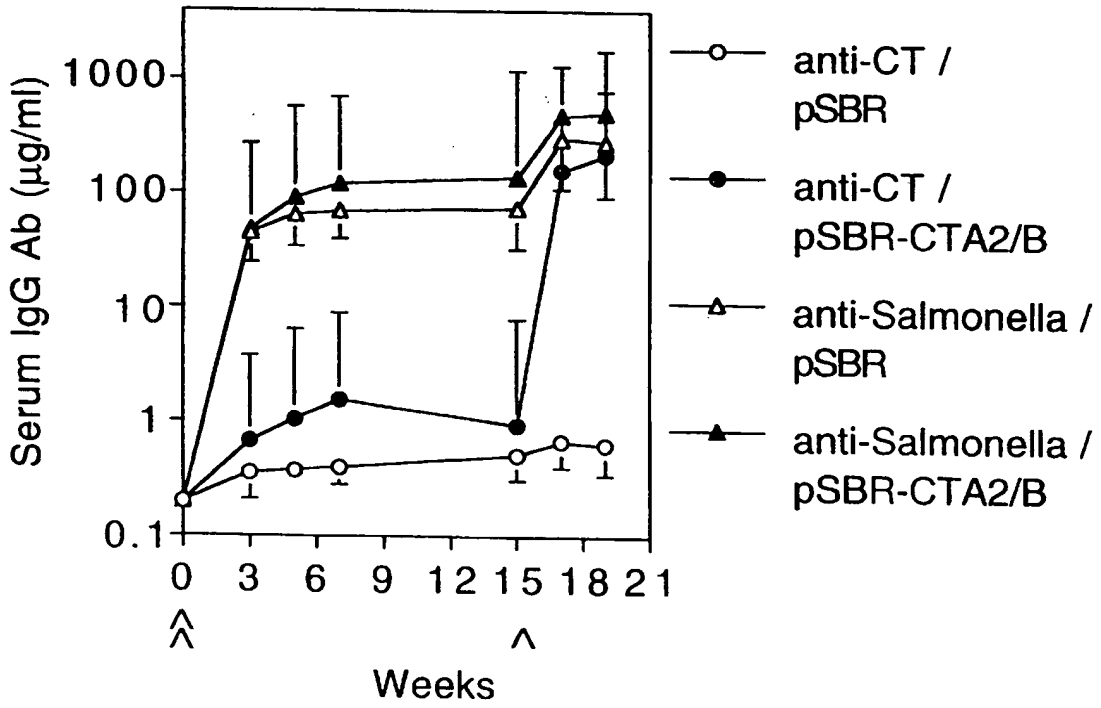

Fig. 10A

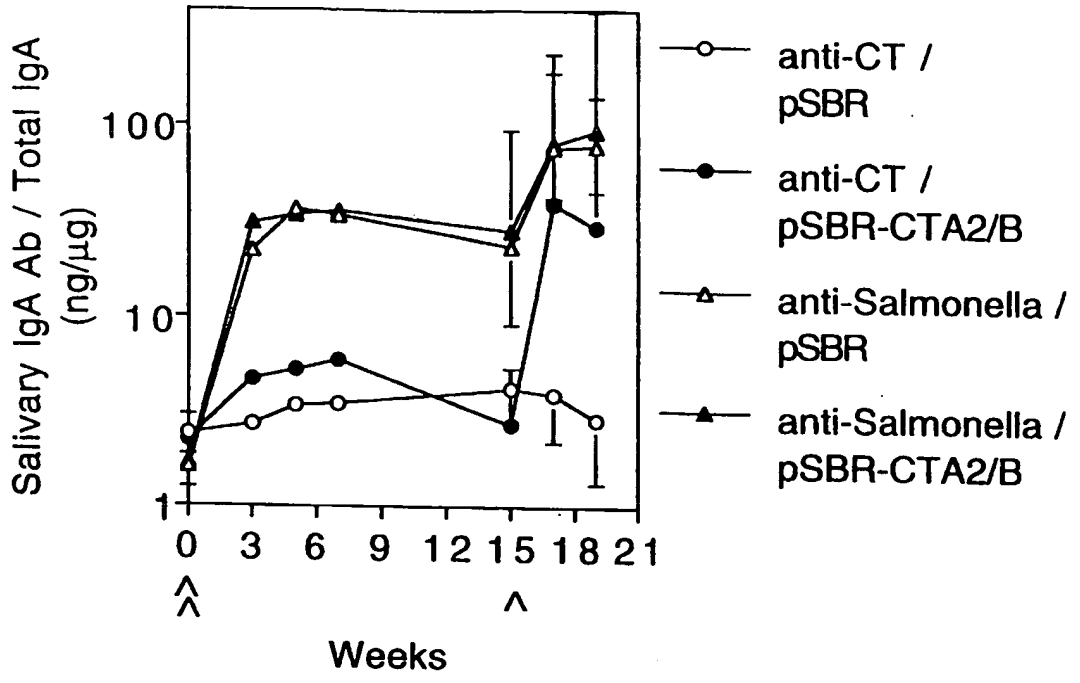

Fig. 10B

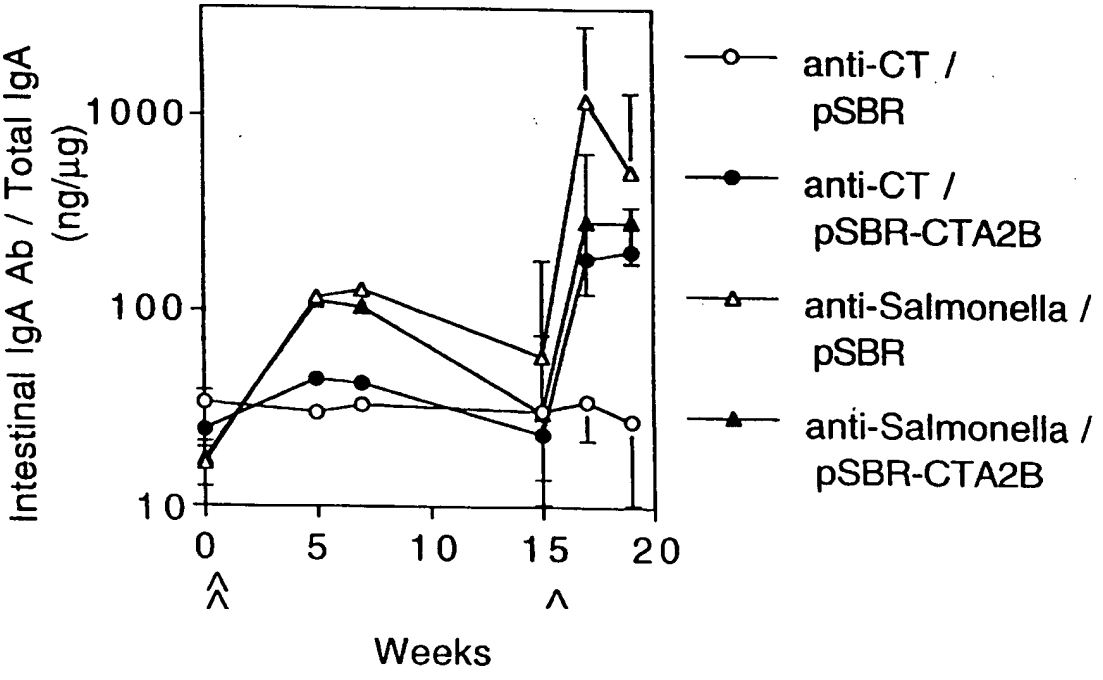

Fig. 10C

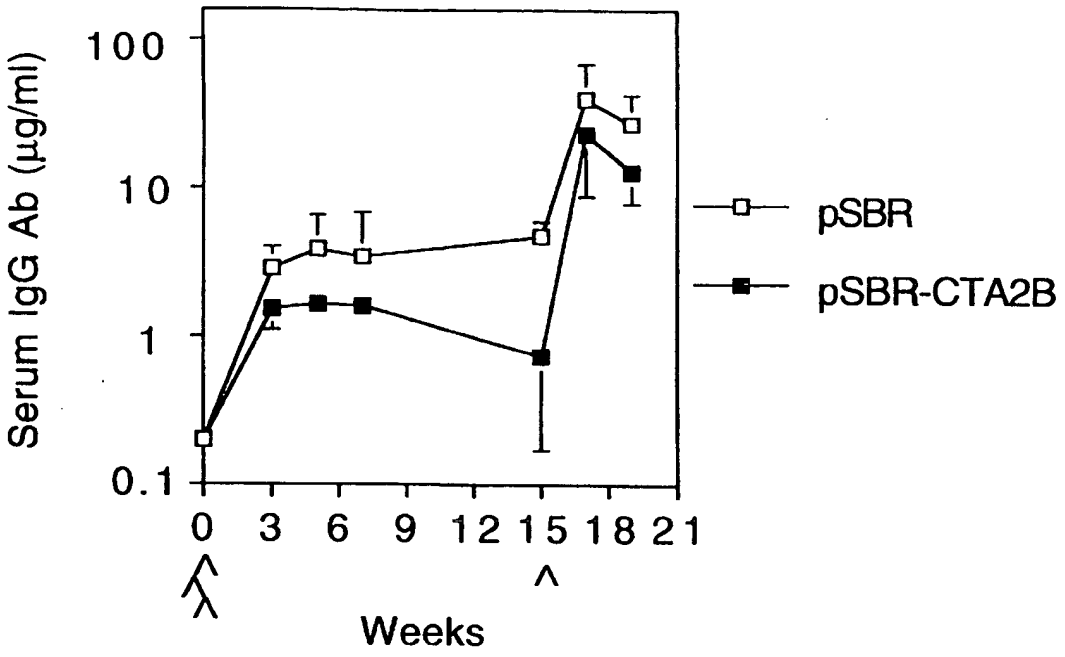

Fig. 11A

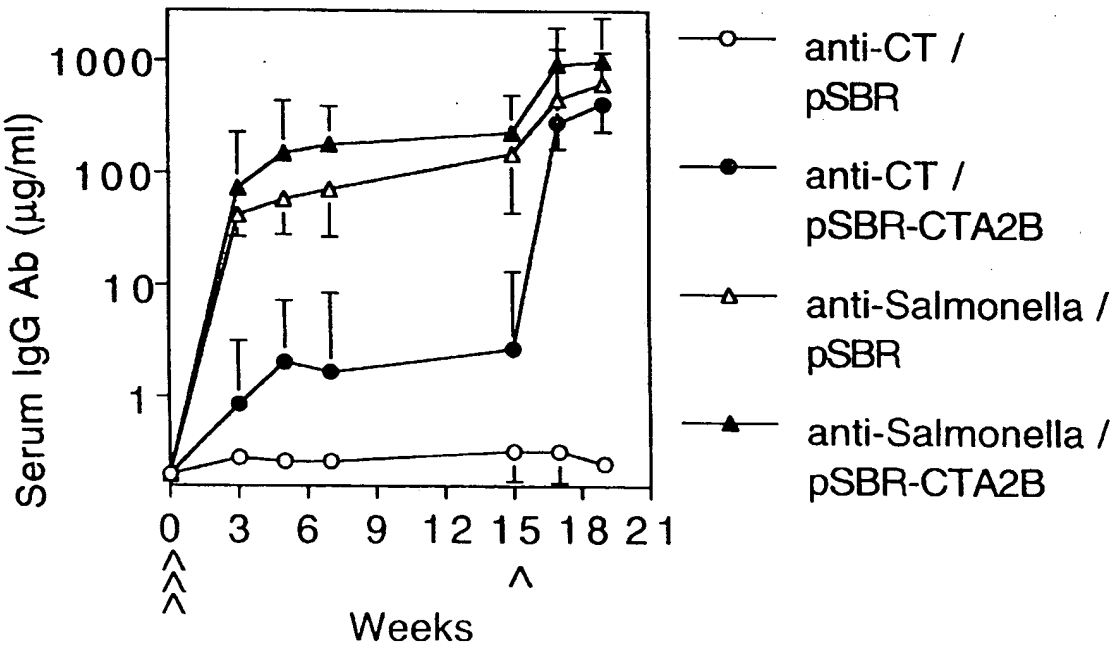

Fig. 11B

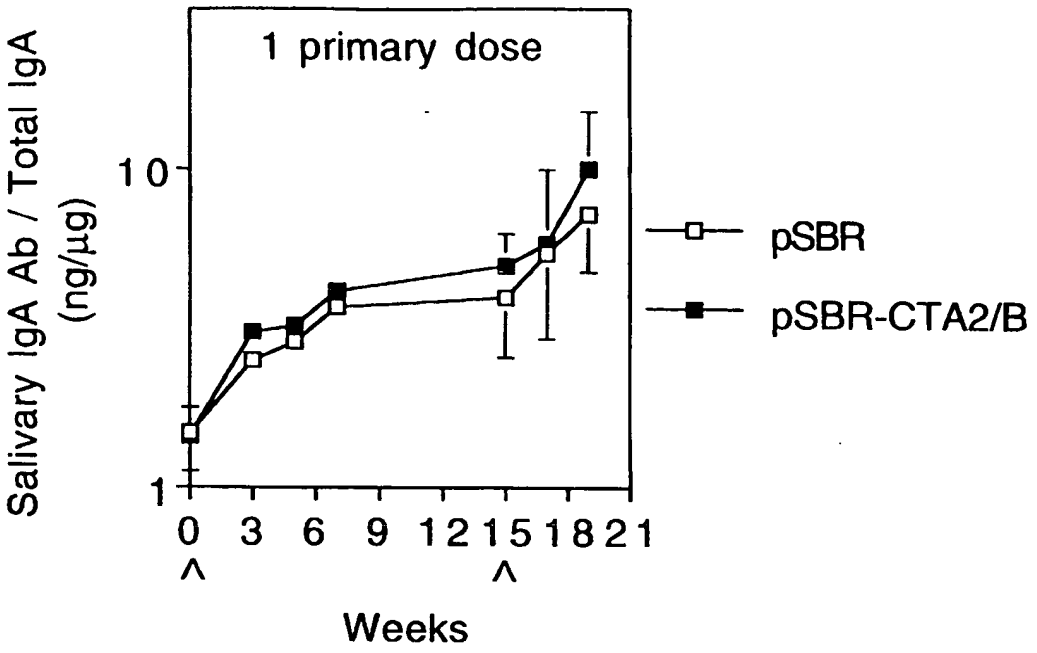

Fig. 12A

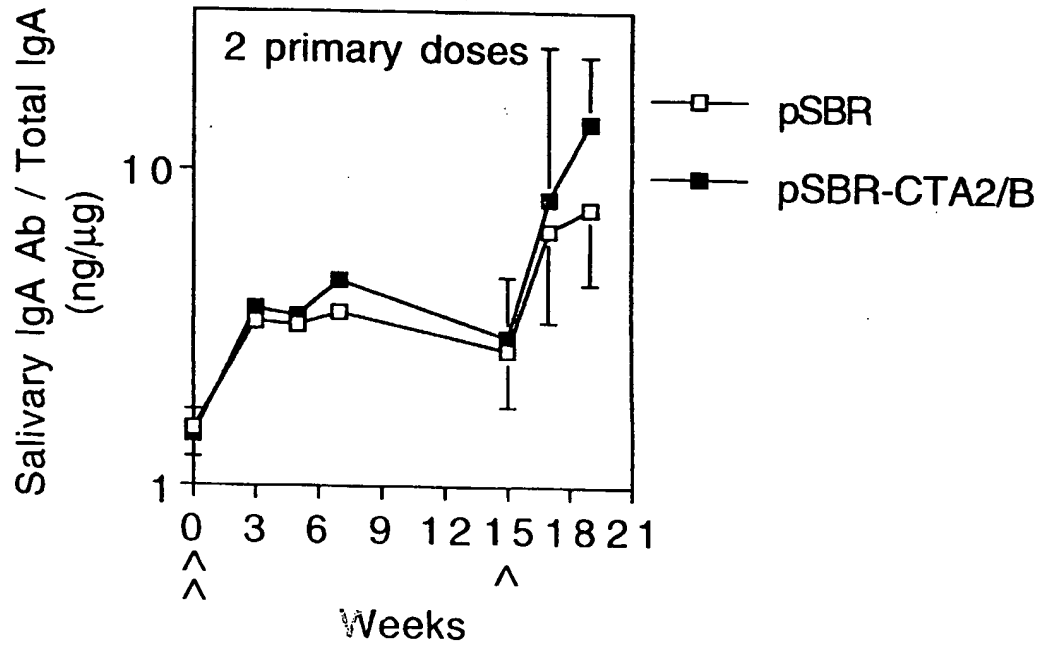

Fig. 12B

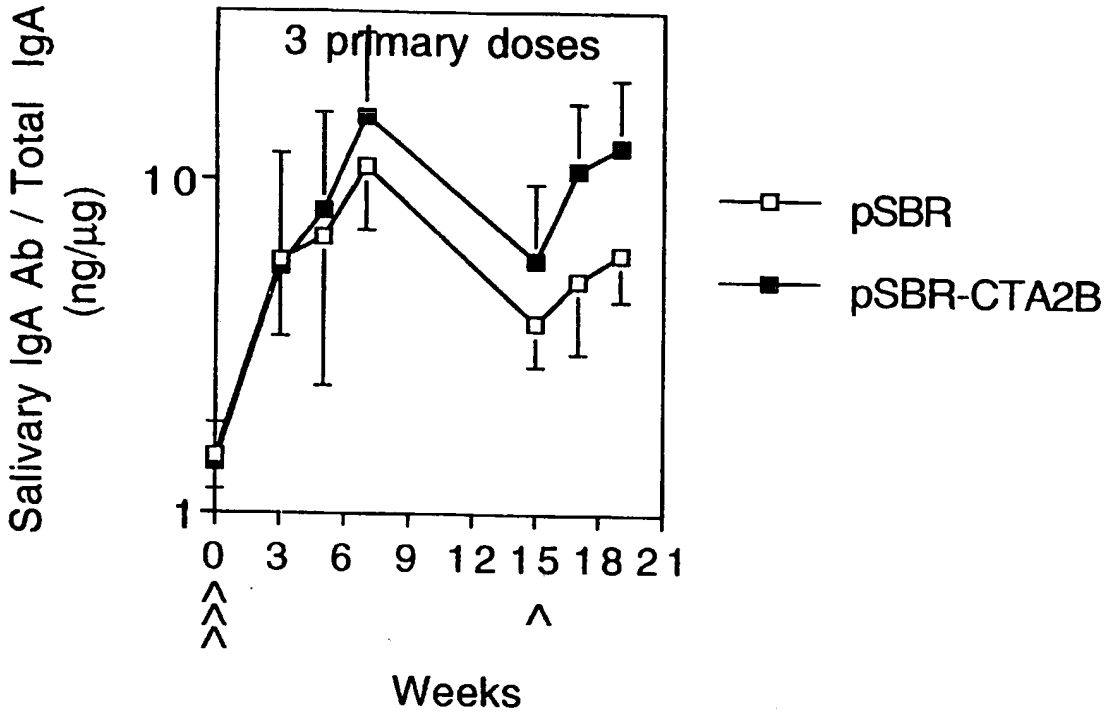
3 primary doses
Salivary IgA Ab / Total IgA (ng/µg)
10
1
0 3 6 9 12 15 18 21
Weeks
pSBR
pSBR-CTA2B

Fig. 12C

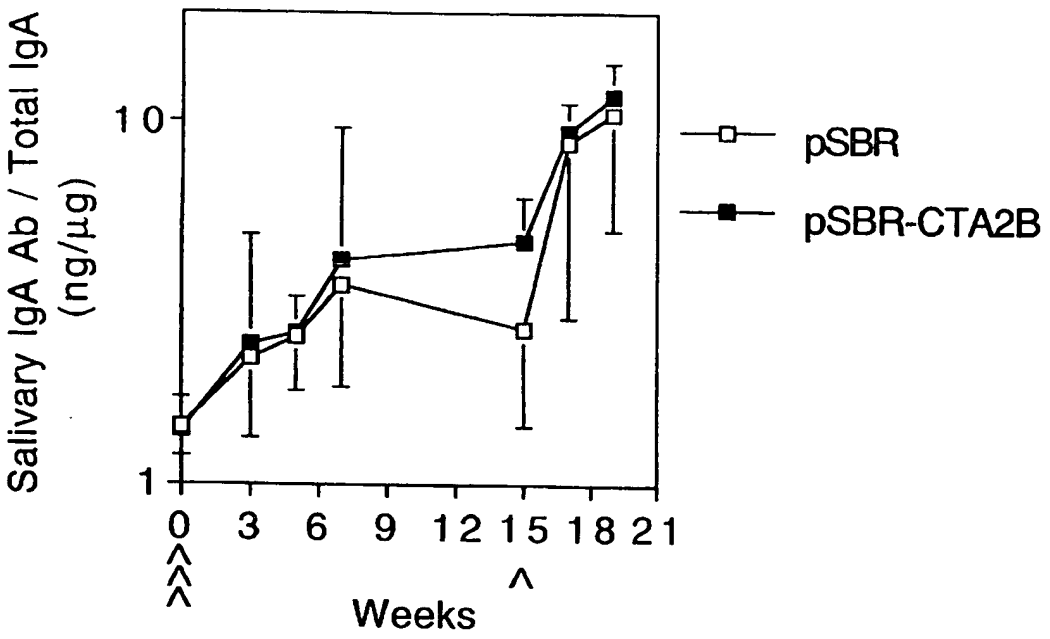

Fig. 13A

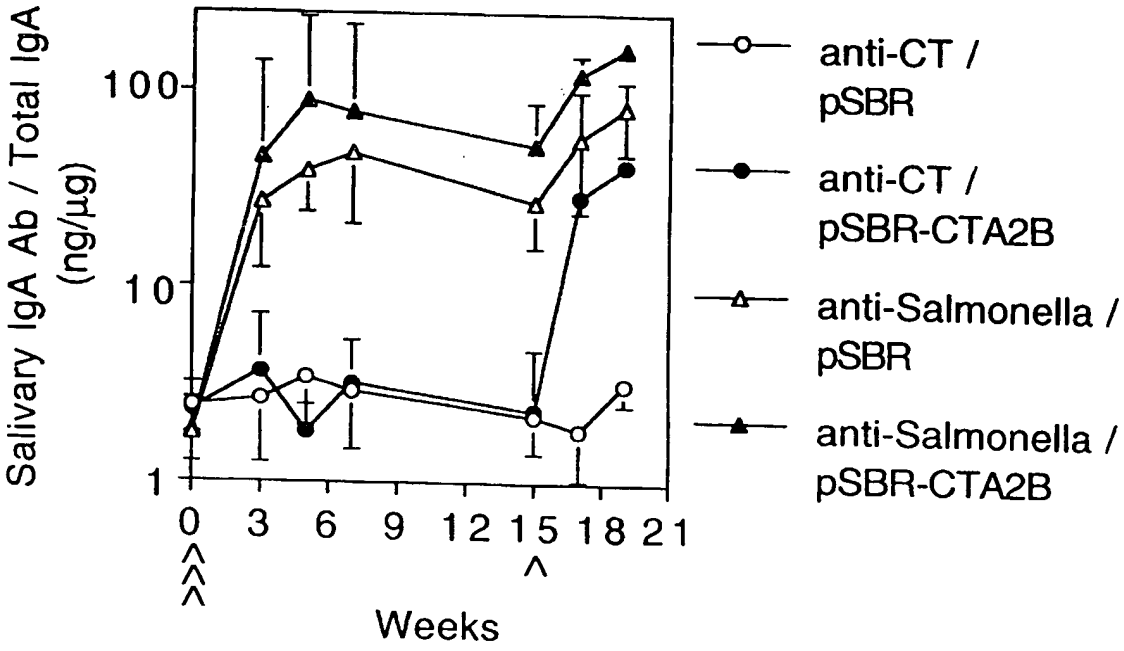

Fig. 13B

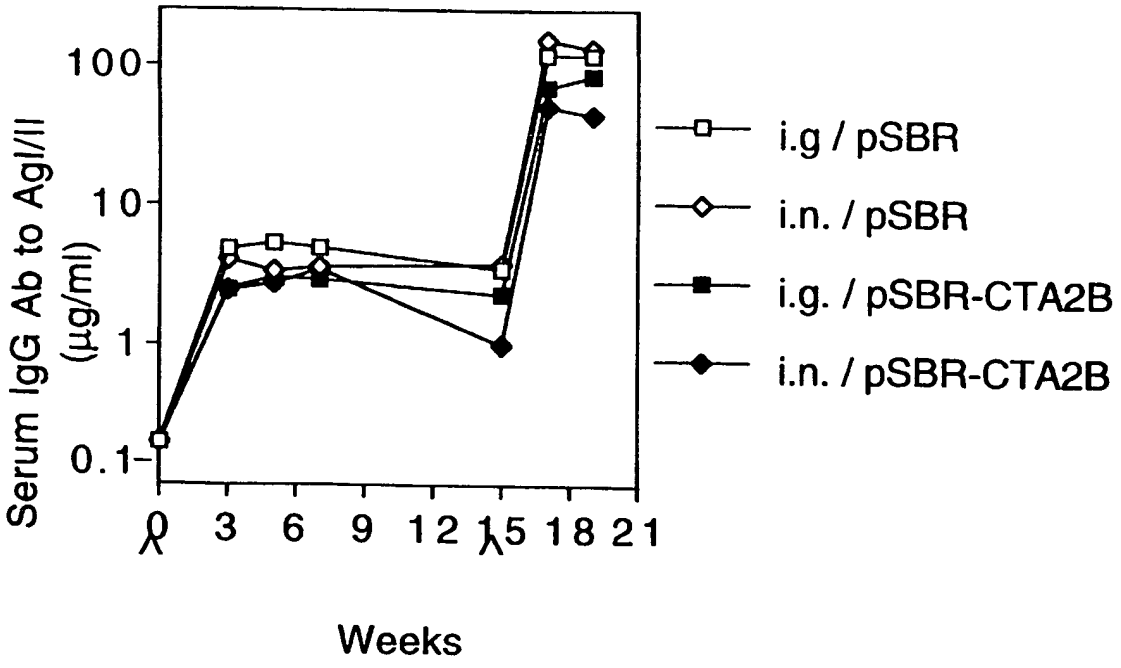

Fig. 14A

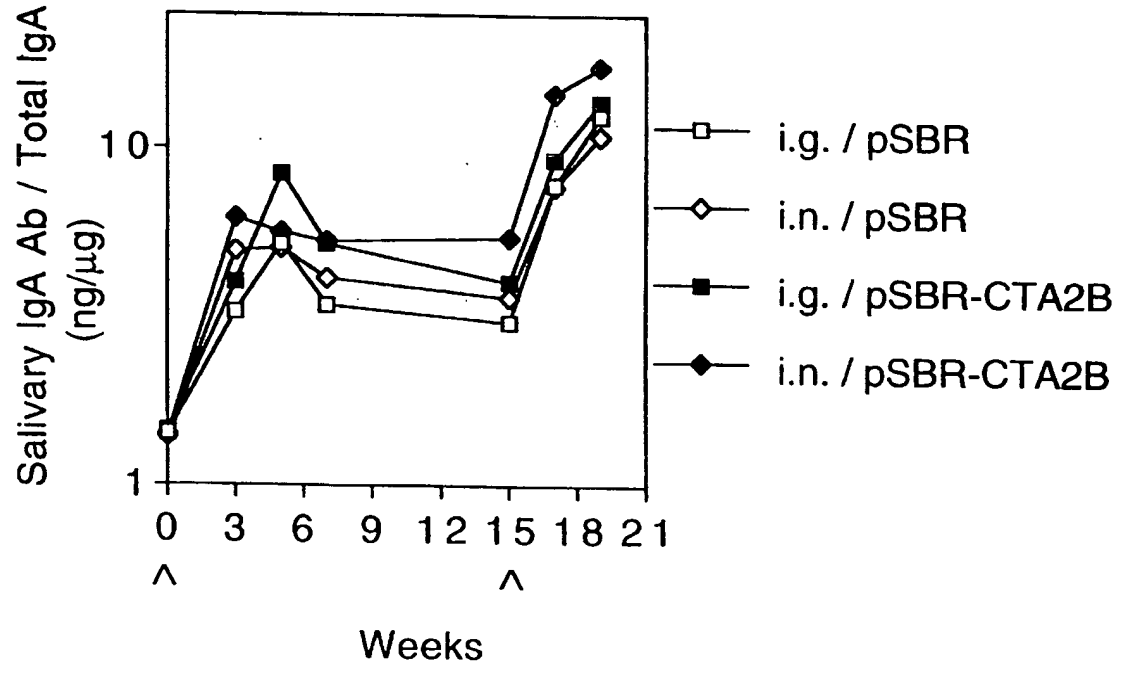

Fig. 14B

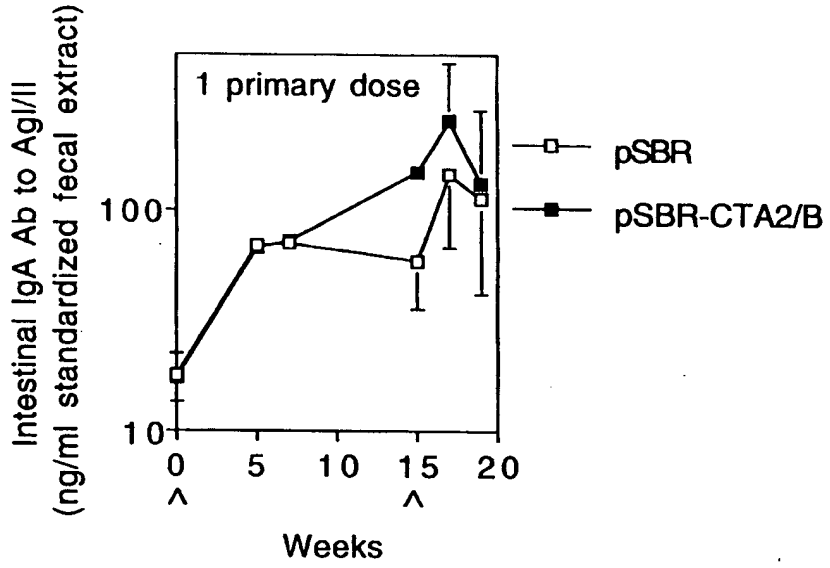

Fig. 15A

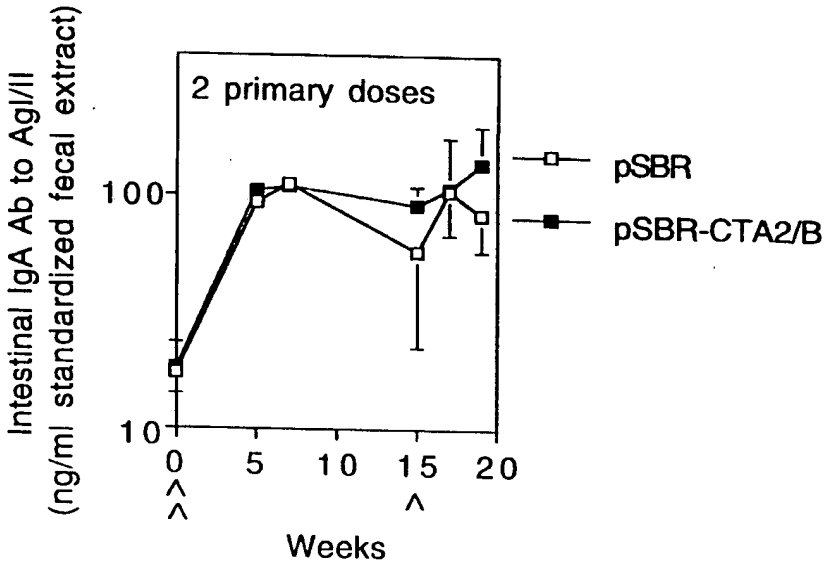

Fig. 15B

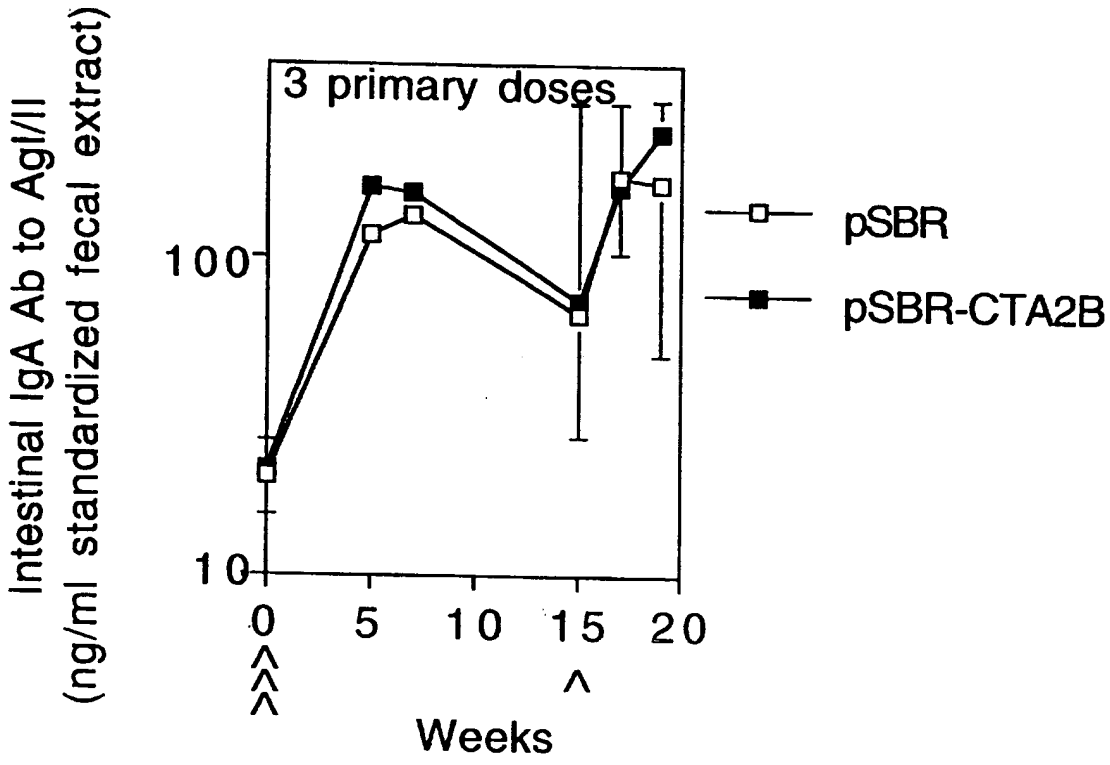
Intestinal IgA Ab to AgI/II
(ng/ml standardized fecal extract)
3 primary doses
100
10
0
5
10
15
20
Weeks
pSBR
pSBR-CTA2B

Fig. 15C

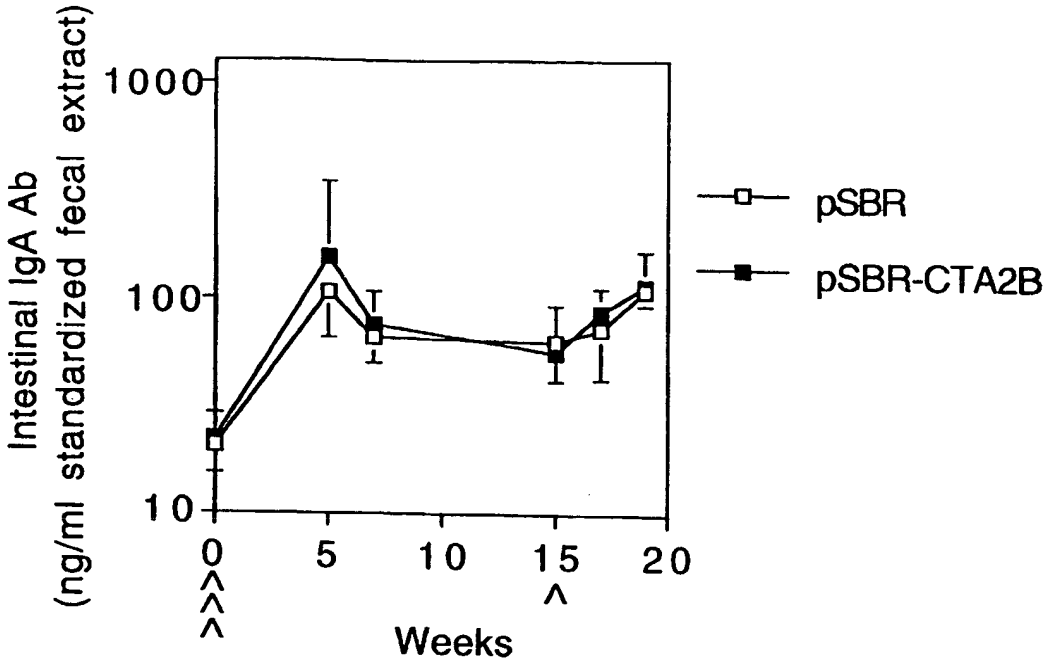

Fig. 16A

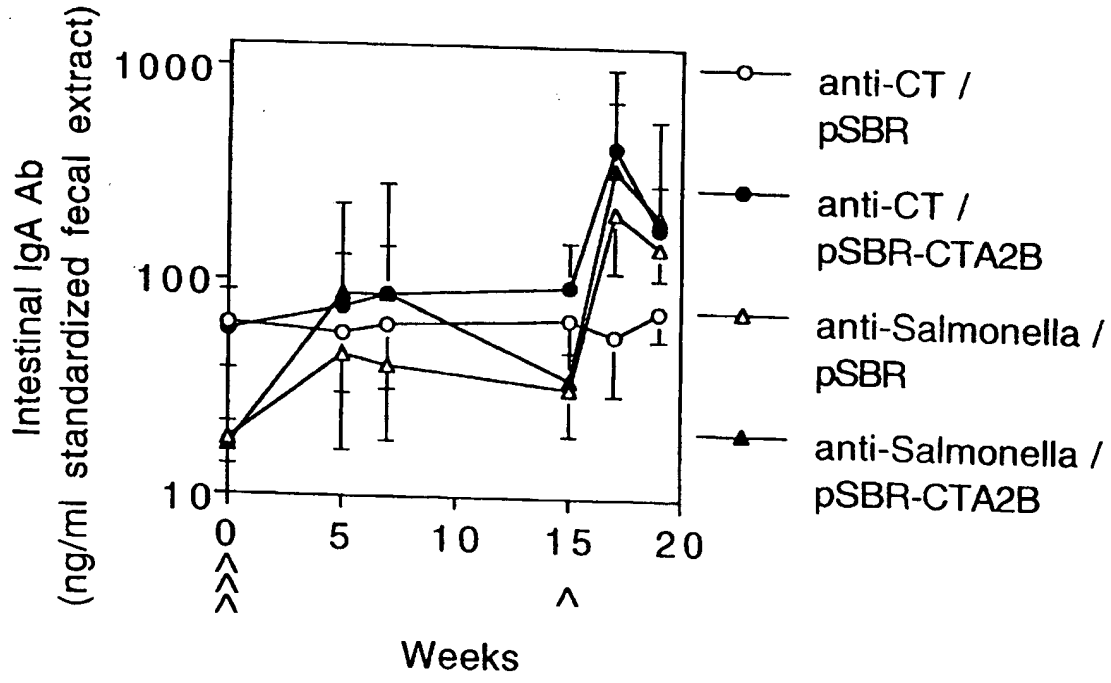

Fig. 16B

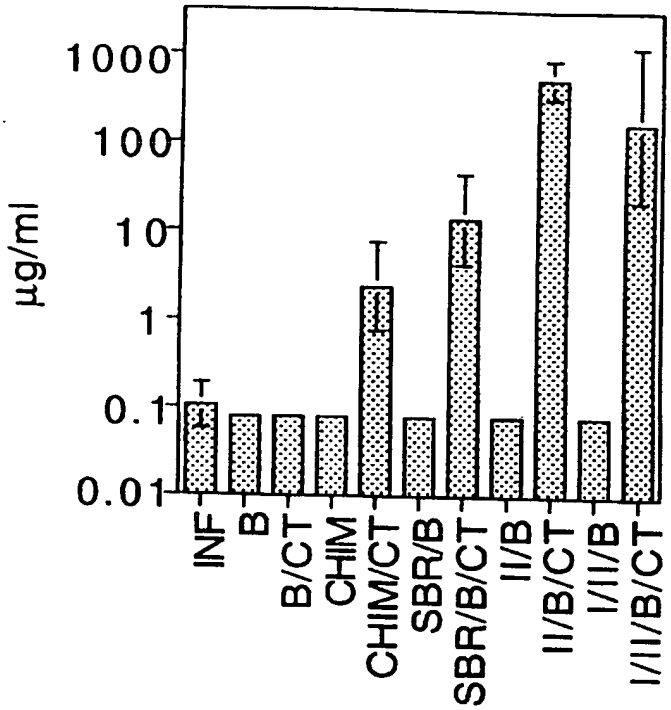
1000
100
10
1
0.1
0.01
µg/ml
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 17

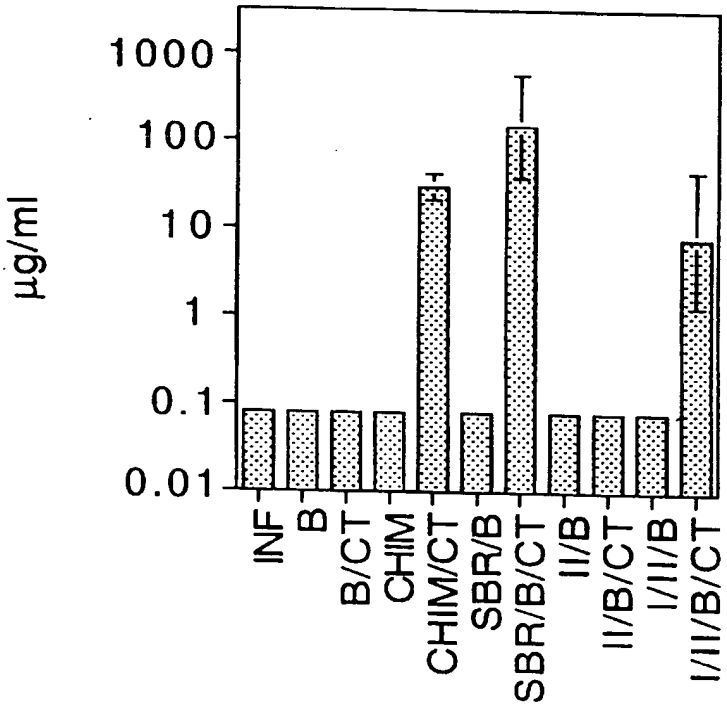
1000
100
10
1
0.1
0.01
µg/ml
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 18

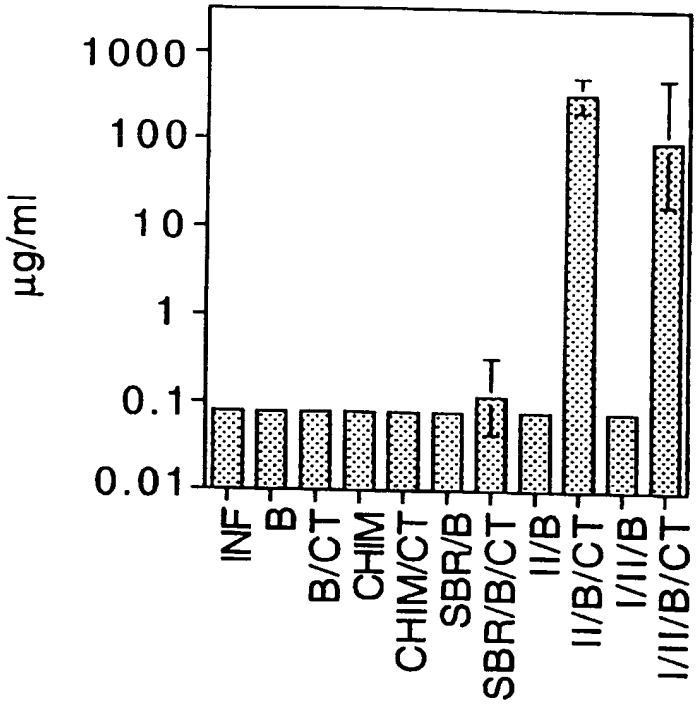
µg/ml
1000
100
10
1
0.1
0.01
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 19

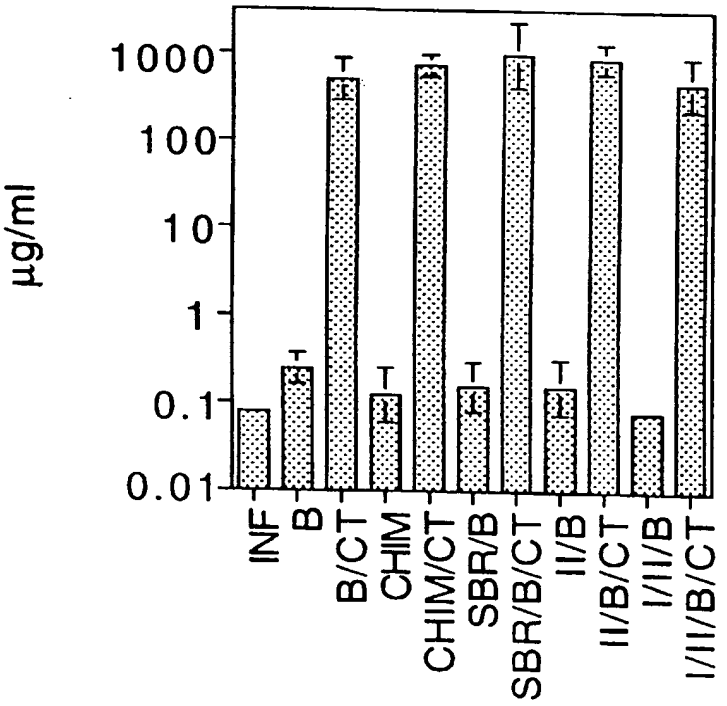
µg/ml
1000
100
10
1
0.1
0.01
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 20

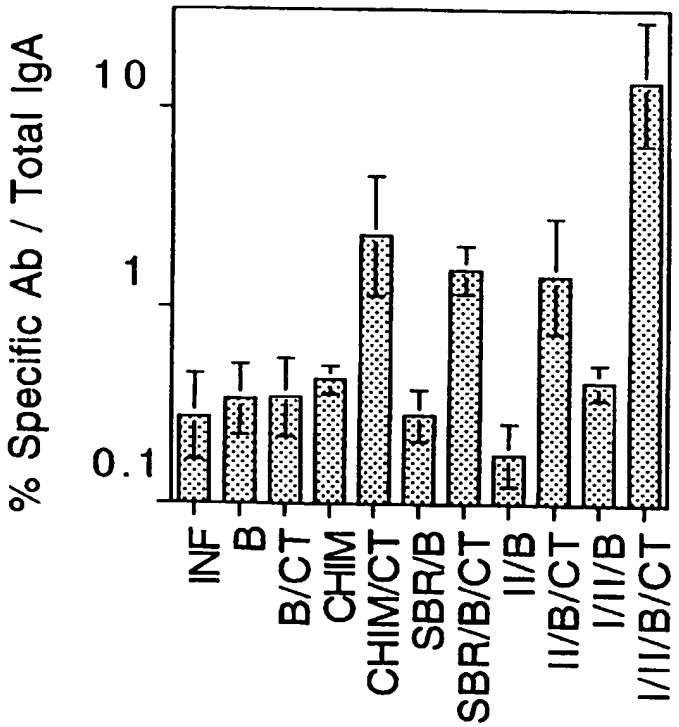
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 21

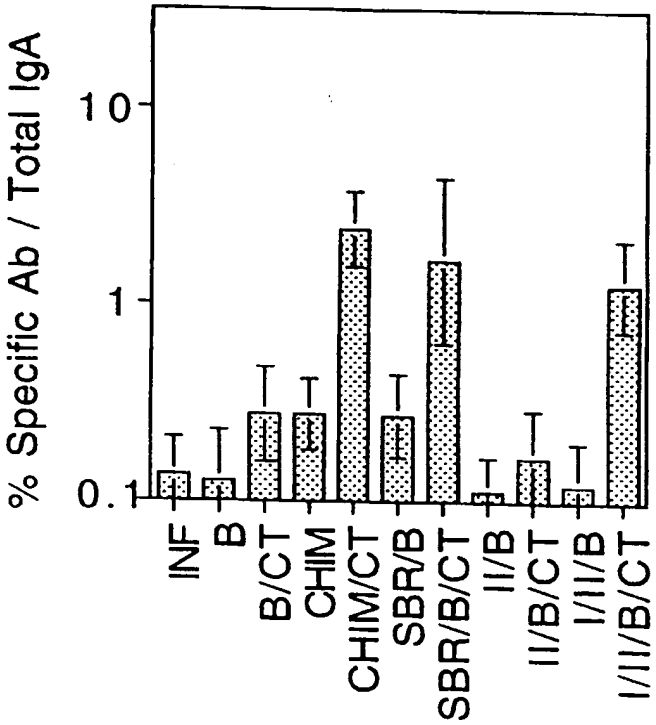
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/II/B
I/II/B/CT

Fig. 22

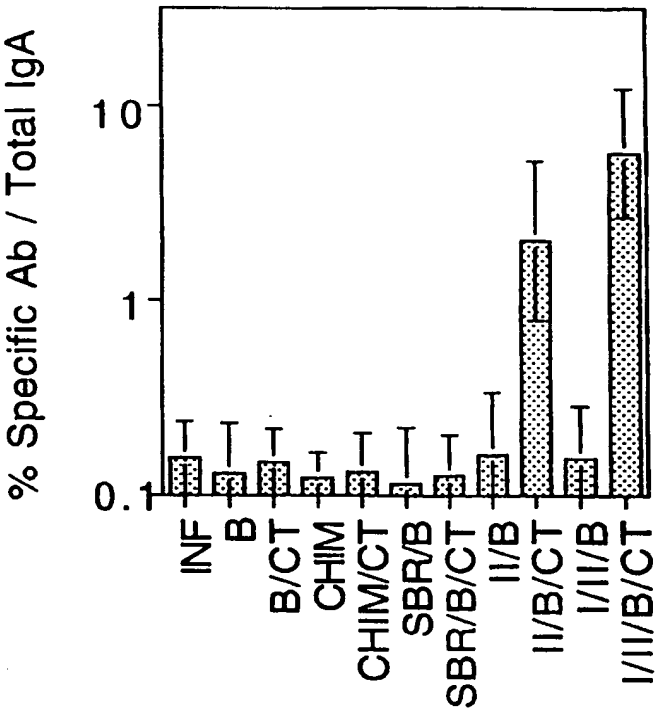
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/III/B
I/II/B/CT

Fig. 23

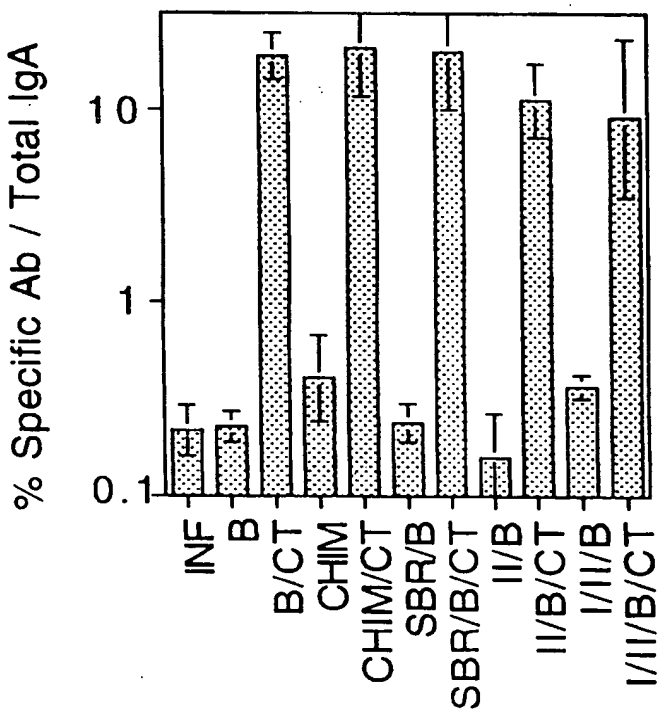
% Specific Ab / Total IgA
10
1
0.1
INF
B
B/CT
CHIM
CHIM/CT
SBR/B
SBR/B/CT
II/B
II/B/CT
I/III/B
I/II/B/CT

Fig. 24

MUCOSAL IMMUNOGENS FOR NOVEL VACCINES

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to provisional application U.S. Ser. No. 60/024,074, filed Aug. 16, 1996, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of molecular immunology and protein chemistry. More specifically, the present invention relates to a novel mucosal immunogens for use in novel vaccines.

2. Description of the Related Art

An oral immunization strategy is when the desired mucosal immunogen is genetically fused to the A2 subunit of cholera toxin (CT) that mediates association with the B subunit of CT, a potent immunoenhancing agent. An antigen selected for evaluating the oral immunogenicity of such non-toxic CTA2/B-based constructs is the saliva-binding region (SBR) of the AgI/II adhesin from the oral bacterium *Streptococcus mutans*. The SBR genetically linked to CTA2/B, designated SBR-CT^{M1}, was found to be immunogenic by the oral route and elicited high levels of secretory immunoglobulin A (S-IgA) and serum IgG antibodies to AgI/II.

Despite its great importance for mucosal defense, the S-IgA antibody response is often of relatively short duration, lasting from a few weeks in experimental animals to a few months in humans. Moreover, whether the secretory immune system is capable of anamnestic immune responses has been debated, but recent studies in mice and humans have addressed the concept of immunological memory at the mucosal surfaces. Immunological memory can be manifested as a long-lasting immune response or as a faster and more vigorous anamnestic response to re-encounter with an antigen. A desirable vaccine characteristic is the induction of prolonged immune responses, especially when the pathogenic organism is frequently encountered at mucosal surfaces, in which case a continuing level of immunity may be necessary.

IgA antibodies in external secretions protect mucosal surfaces, e.g., of the gastrointestinal and respiratory tracts, by blocking microbial adherence and colonization. Oral administration of vaccines can result in the induction of secretory immune responses after uptake of the antigen by the gut-associated lymphoid tissues, a major IgA inductive site. However, most soluble proteins are not only poor immunogens when given orally but they may induce a state of systemic unresponsiveness known as "oral tolerance". The experimental use of cholera toxin from *Vibrio cholerae* or the related heat-labile enterotoxin from *Escherichia coli* as mucosal adjuvants inhibit induction of oral tolerance and potentiates the immune responses to co-administered protein antigens.

Another strategy to overcome problems associated with oral immunization (e.g., denaturation of the protein immunogens by gastric acid and digestive enzymes, limited absorption by the intestinal mucosa, and clearance by peristalsis) as well as the need to purify a vaccine protein, involves the use of avirulent derivatives of *Salmonella typhimurium* as a vaccine delivery system with tropism for the gut-associated lymphoid tissues. Oral immunization with avirulent *S. typhimurium* expressing heterologous antigens

is generally not associated with suppression but rather with stimulation of protective secretory and serum antibody responses as well as cell-mediated immune responses.

Initial adherence of *Streptococcus mutans* to tooth surfaces appears to be mediated largely by the 167 kDa surface fibrillar adhesin known as AgI/II (synonyms: antigen B, P1, SpaP, PAc). The adhesion domain that interacts with salivary pellicle has been located to the alanine-rich (A) repeat region in the N-terminal part of the molecule extending from the cell surface probably in an α-helical conformation. Studies on AgI/II indicated that rhesus monkeys immunized with *S. mutans* and protection against dental caries mounted antibody responses especially against the complete molecule rather than against AgII, which corresponds to the C-terminal one-third. These results were supported by the finding that immunization with either complete AgI/II, or the isolated AgI component (corresponding to the N-terminal two-thirds), afforded protection against caries. Thus, one approach to immunization against *S. mutans*-induced dental caries can be based upon the generation of an appropriate antibody response in the saliva that would inhibit the adherence of *S. mutans* to tooth surfaces. Human secretory IgA (S-IgA) antibodies to AgI/II inhibit such adherence. However, S-IgA antibodies in saliva and other secretions are not effectively induced by conventional parenteral immunization.

S-IgA antibodies are most effectively induced by stimulating the common mucosal immune system, for example, by enteric immunization which stimulates the gut-associated lymphoid tissues including the Peyer's patches (PP) of the small intestine. Considerable attention has been given to the development of improved procedures for the oral delivery of vaccines, one of which is coupling antigens to the nontoxic binding B subunit of cholera toxin (CT), a safe and highly immunogenic protein in humans. CTB, because of its avid binding to G_{M1} ganglioside, present on all nucleated cell surfaces, is readily taken up by the M cells covering PP, and passed to the underlying immunocompetent cells which initiate the mucosal IgA antibody response. Antigen-stimulated IgA-committed B cells, and corresponding T helper cells, then emigrate via draining lymphatics to the mesenteric lymph nodes (MLN) and thence via the thoracic duct to the circulation before relocating in the effector sites of mucosal immunity, such as the salivary glands. Terminal differentiation of B cells into IgA-secreting plasma cells occurs here and their product, polymeric IgA is transported through the glandular epithelium to form S-IgA. Other antigens can be coupled to CTB to generate strong mucosal IgA antibody responses to the desired antigen and that intact CT, though toxic, serves as an adjuvant that enhances the response to co-administered antigens.

The expression of foreign genes encoding immunogens of interest in avirulent derivatives of *Salmonella typhimurium* is used as a strategy to induce mucosal immune responses to protein Ags which are usually poor oral immunogens when administered alone. Indeed, *S. typhimurium* appears to be an effective antigen delivery system because of its ability to colonize the gut-associated lymphoid tissue where secretory IgA responses are initiated (1). Electron microscopy studies have shown that *S. typhimurium* preferentially interacts with the specialized antigen-sampling M cells overlying the Peyer's patches in the GALT (2). At these sites, antigenic stimulation of specific IgA-committed B cells results in their migration to mucosal tissues where they differentiate into IgA-secreting plasma cells, with subsequent release of secretory IgA antibodies in external secretions (3). These antibodies play an important role in the defense of mucosal

surfaces, e.g., of the gastrointestinal and respiratory tracts, by inhibiting microbial adherence and colonization or invasion (4). Depending on the species and host, Salmonella organisms may disseminate to the spleen, the liver, and regional lymph nodes, take residence in macrophages, and thereby induce serum antibody and cellular immune responses (1).

The issue of whether CTB alone has mucosal adjuvant properties has been questioned especially for oral immunization (14, 19), although CTB confers a targeting property to Ags coupled to it because of its affinity for G_{M1} ganglioside receptors (20). If CTB possesses immunoenhancing properties, other than its carrier/targeting effect, it could also be useful as a Salmonella-expressed adjuvant, especially for proteins that are poor immunogens even when delivered by *S. typhimurium*. A commercially obtained CTB preparation, lacking detectable cAMP-elevating capacity, was found to potentiate in vitro antibody production against an unrelated protein antigen by stimulating the antigen-presenting function of splenic adherent cells through enhanced IL-1 production (21). An enhancing effect on antigen presentation by macrophages was also demonstrated for recombinant (r)CTB (22), which, moreover, up-regulates expression of MHC class II molecules on B cells, which can also act as antigen-presenting cells (23). The fact that commercially available CTB is contaminated with small but variable amounts of intact CT may explain conflicting reports on the adjuvant capacity of CTB (14) as well as findings that commercial CTB is superior to rCTB as an adjuvant for intranasal (i.n.) immunization (24, 25).

The prior art is deficient in the lack of effective mucosal immunogens, for use in, e.g., a caries vaccine. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention demonstrates that primary oral immunization of mice with a bacterial protein antigen genetically coupled to the A2/B subunits of cholera toxin induced specific secretory immunoglobulin A and serum IgG antibodies that persisted at substantial levels for at least 11 months. A subsequent single booster immunization did not further enhance the antibody responses. Long-term antibody persistence may be especially important in infections caused by common pathogens for which continuous immunity would be advantageous.

The present invention further shows that a major adhesin from the oral pathogen *Streptococcus mutans* is mucosally immunogenic upon genetic fusion with the cholera toxin A2/B subunits. To take advantage of the ability of *Salmonella typhimurium* to deliver cloned antigens to the mucosal inductive sites that would obviate the need for antigen purification, this chimeric construct was expressed in an attenuated *S. typhimurium* strain under the control of bacteriophage T7 transcription. Residual expression of the temperature-regulated T7 RNA polymerase at 30° C. allowed production of the chimeric protein at 2–3% of the total soluble protein, but it was increased 5–6 times following induction at 37° C. Oral administration of a single dose of 10^9 recombinant Salmonella to mice resulted in serum IgG and salivary IgA antibody responses to Salmonella, cholera toxin, and the streptococcal adhesin, which were generally enhanced after a booster immunization.

The present invention also discloses an avirulent *Salmonella typhimurium* vaccine strain expressing a streptococcal protein adhesin, and a similar clone which produces the same streptococcal antigen linked to the cholera toxin A2/B subunits, which were compared for their ability to induce antibody responses to the expressed heterologous antigen after oral or intranasal immunization of mice. Expression of cloned immunogens in these systems is temperature-regulated, being optimal at 37° C., and the two clones produced similar levels of the streptococcal antigen. Both clones were found to stimulate high levels of serum IgG and mucosal IgA antibodies to the cloned immunogen. A consistent trend was observed towards higher mucosal IgA but lower serum IgG responses in the case of the *S. typhimurium* vector that co-expressed CTA2/B, a potential mucosal adjuvant, regardless of the route of administration. Also noteworthy was the capacity of these antigen-delivery systems to induce anamnestic mucosal and systemic responses to the cloned immunogen 15 weeks after the primary immunization, despite pre-existing immunity to the Salmonella vectors. Although the serum IgG response against the Salmonella vector was characterized by a high IgG2a/IgG1 ratio (indicative of the Th1/Th2 profile), a mixed IgG1 and IgG2a pattern was observed for the carried heterologous antigen, which displayed a dominant IgG1 response when administered as a purified immunogen. The present invention indicates that the recombinant streptococcal antigen and CTA2/B are strong immunogens when expressed by the antigen-delivery system, and that CTA2/B may have an additional immunoenhancing activity in the mucosal compartment besides its ability to target antigen uptake into the mucosal inductive sites and, therefore, may be useful as a *S. typhimurium*-cloned adjuvant for co-expressed protein Ags.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

FIGS. 1(A)–1(D) shows the persistence of serum IgG antibody to AgI/II and CT after peroral immunization of mice with SBR-CT^{AA1} chimeric protein (~~) and a single booster immunization 11 months later (^). Mice were given the immunogen in the presence (FIG. 1A and FIG. 1C) or absence FIG. 1B and FIG. 1D of CT adjuvant. Data are presented for each mouse individually.

FIGS. 2(A)–2(D) shows the duration of salivary IgA antibody to AgI/II and CT following peroral immunization of mice with SBR-CT^{AA1} chimeric protein (~~) and a single booster immunization 11 months later (^). Mice were given the immunogen in the presence FIG. 2A and FIG. 2C or absence FIG. 2B and FIG. 2D of CT adjuvant. Data are presented for each mouse individually.

FIG. 3 shows the SDS-PAGE analysis of inclusion bodies produced under different induction conditions by *S. typhimurium* expressing the SBR-CT^{AA1} chimeric protein. A related strain lacking SBR-CT^{AA1} was used as a negative control. Two prominent bands migrating at about 60 kDa and about 14 kDa present in the expressing strain after 42° C. induction (and to a lesser extent after 37° C. induction)

correspond to the Mr of SBR-CTA2 and CTB (monomer), respectively. The CTB component of purified SBR-CT$^{\Delta A1}$ ran relatively faster (Mr~11.5 kDa) due to processing of the precursor polypeptide by signal peptidase during transport to the periplasmic space, while an additional band from SBR-CTA2 may represent a degradation product.

FIGS. 4(A)–4(B) shows the serum IgG FIG. 4A and salivary IgA FIG. 4B antibody responses to *S. typhimurium* and cloned antigens in mice orally immunized with 10^9 bacteria on weeks 0 and 7 (^). Immune response data for weeks 5, 8 and 10 represent geometric means ×/+ standard deviation of five mice. Pooled samples were assayed at other time points.

FIGS. 5(A)–5(B) show the serum IgG FIG. 5A and salivary IgA FIG. 5B antibody responses to AgI/II in unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Immunizations were given on days 0, 10, and 20, and samples were collected 10 days after each immunization, i.e., on days 0 (unimmunized mice), 10 (one dose), 20 (2 doses), or 30 (3 doses). Results shown are mean+/–SD of samples from 3 animals analysed separately. Salivary IgA antibodies were below detectable levels (<0.1 μg/ml) on days 0 and 10, and are shown at this level in (b).

FIG. 6 shows the proliferative responses of cells from PP, MLN, and spleens of unimmunized (control) mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant, cultured in vitro with AgI/II. Results shown are mean stimulation indices of 3 replicate cultures; SD values ranged from ~0.04 to ~0.95.

FIG. 7 shows the phenotypic analysis of cells from PP, MLN, peripheral blood, and spleen of unimmunized mice and mice immunized once, twice, or three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT adjuvant. Each 'pie' shows the proportions of $CD4^+$, $CD8^+$, and $CD3^-$ (non-T) cells as a percentage of total gated mononuclear cells determined by flow cytometry, starting with unimmunized mice (center of each 'pie'), and proceeding outwards in concentric rings with mice immunized once, twice, or three times. Numbers within the rings are the % of each phenotype of cells (for clarity, $CD8^+$ cell data are shown outside the 'pies' in descending order: 0, 1, 2, and 3 doses); the value shown for MLN from mice immunized once with SBR (marked as "51?") was not obtained experimentally, but was inserted for plotting purposes as the average of the values either side of it. The numbers in each ring of a 'pie' do not sum to exactly 100%, because of the presence of some $CD4^-/CD8^-$ ("double-negative") $CD3^+$ T cells, and possibly some $CD4^+/CD8^+$ ("double-positive") T cells in each cell preparation.

FIG. 8A shows a schematic representation of the plasmids used to transform SBR-CT$^{\Delta A1}$-or SBR-expressing *S. typhimurium* clones. FIG. 8B shows a western blotting of cell lysates from the SBR-CT$^{\Delta A1}$-and the SBR-producing clones using antibodies to SBR detected the SBR-CTA2 fusion protein and the SBR, respectively. The control lane is a lysate from a clone transformed with the pGP1-2 plasmid only.

FIGS. 9(A)–9(C) shows the serum IgG antibody responses to native AgI/II in mice orally immunized (^) on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* clones. During week 0 mice were immunized with one FIG. 9A, two FIG. 9B, or three FIG. 9C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Data represent geometric means ×/(SD of 5 to 6 mice. For clarity only the upper or lower SD bars are shown.

FIGS. 10(A)–10(C) shows the serum IgG (FIG. 10A), salivary IgA (FIG. 10B), and intestinal IgA (FIG. 10C) antibody responses to CT and *S. typhimurium* vector after oral immunization (^) of mice with SBR- or SBR-CT$^{\Delta A1}$-producing Salmonella clones on weeks 0 and 15. Mice were immunized with two doses of 10^9 CFU of the appropriate *S. typhimurium* clone during week 0, and were given a single dose of 10^{10} CFU 15 weeks later. Results are shown as geometric means ×/+ SD of 5 to 6 mice. Data for weeks 3 to 7 (FIGS. 10B and 10C) were obtained by assaying pooled samples from 5 to 6 mice per corresponding group. For clarity only the upper or lower SD bars are shown.

FIGS. 11(A)–11(B) shows the serum IgG antibody responses to AgI/II (FIG. 11A) and CT and Salmonella vector (FIG. 11B) in mice immunized (^) by the i.n. route on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S.typhimurium* clones. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data are expressed as the geometric means ×/(SD of 6 mice.

FIGS. 12(A)–12(C) shows the salivary IgA antibody responses to AgI/II in mice orally immunized (^) on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S.typhimurium*. During week 0 mice were immunized with one FIG. 12A, two FIG. 12B, or three FIG. 12C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Results are the geometric means ×/(SD of 5 to 6 mice. Data for weeks 3, 5, and 7 in groups which received one or two primary doses, were obtained by assaying pooled samples from 5 to 6 mice per corresponding group.

FIGS. 13(A)–13(B) shows the salivary IgA antibody responses to AgI/II (FIG. 13A) and CT and Salmonella vector (FIG. 13B) after i.n. immunization (^) of mice on weeks 0 and 15 with SBR- or SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* clones. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data represent geometric means ×/(SD of 6 mice.

FIGS. 14(A)–14(B) shows the serum IgG FIG. 14A and salivary IgA FIG. 14B antibody levels to AgI/II induced by either i.n. or i.g. immunization (^) of mice on weeks 0 and 15 with *S. typhimurium* expressing SBR alone or SBR-CT$^{\Delta A1}$ chimeric protein. The animals were given a single primary immunization of 10^9 CFU (i.n.) or 10^{10} CFU (i.g.) followed by a booster immunization with the same dose 15 weeks later. Data were obtained by assaying pooled samples from 3 mice per corresponding group.

FIGS. 15(A)–15(C) shows the intestinal IgA antibody responses to AgI/II in mice orally immunized (^) on weeks 0 and 15 with *S. typhimurium* clones expressing SBR or SBR-CT^{DA1}. During week 0 mice were immunized with one FIG. 15A, two FIG. 15B, or three FIG. 15C doses of 10^9 CFU of the appropriate *S. typhimurium* clone. At 15 weeks the animals were given a single dose of 10^{10} CFU. Results are the geometric means ×/(SD of 5 to 6 mice. Data for weeks 5 and 7 were obtained by assaying pooled samples from 5 to 6 mice per corresponding group.

FIGS. 16(A)–16(B) shows the intestinal IgA antibody responses to AgI/II (FIG. 16A) and CT and *S. typhimurium* (FIG. 16B) after i.n. immunization (^) of mice on weeks 0 and 15 with *S.typhimurium* vectors producing SBR alone or

SBR-CT$^{\Delta A1}$ chimeric molecule. During week 0 mice were immunized with three doses of 10^8 CFU of the appropriate *S. typhimurium* clone and 15 weeks later they were given a single dose of 10^9 CFU. Data represent geometric means ×/(SD of 6 mice.

FIG. **17** shows the serum IgG antibody responses to native AgI/II in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of recombinant B subunit of cholera toxin (rCTB) or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of cholera toxin (CT). Data are from samples obtained two weeks after the last immunization and represent geometric means±standard deviation (SD).

FIG. **18** shows the serum IgG antibody responses to recombinant SBR (42-kDa saliva-binding region at the N-terminal of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definitions) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **19** shows the serum IgG antibody responses to AgII (C-terminal one-third of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent means±SD.

FIG. **20** shows the serum IgG antibody responses to CT in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **21** shows the salivary IgA antibody responses to native AgI/II in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of recombinant B subunit of cholera toxin (rCTB) or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of cholera toxin (CT). Data are samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **22** shows the salivary IgA antibody responses to recombinant SBR (42-kDa saliva-binding region at the N-terminal of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are samples obtained two weeks after the last immmunization and represent geometric means±SD.

FIG. **23** shows the salivary IgA antibody responses to AgII (C-terminal one-third of AgI/II) in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

FIG. **24** shows the salivary IgA antibody responses to CT in intranasally immunized rats. The animals were immunized three times at 14-day intervals with 25 μg of rCTB or with 50 μg of the appropriate immunogen genetically or chemically linked to rCTB (see group definition) in the presence or absence of an adjuvant amount (1 μg) of CT. Data are from samples obtained two weeks after the last immunization and represent geometric means±SD.

Group Abbreviations	Groups Defined
INF	Unimmunized controls
B	rCTB
B/CT	rCTB + CT
CHIM	SBR-CT$^{\Delta A1}$
CHIM/CT	SBWCT$^{\Delta A1}$ + CT
SBR/B	SBR-rCTB
SBR/B/CT	SBR-rCTB + CT
II/B	AgII-rCTB
II/B/CT	AgII-rCTB + CT
I/II/B	AgI/II-rCTB
I/II/B/CT	AgI/II-rCTB + CT

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations may be used herein: GALT: gut-associated lymphoid tissue; CT: cholera toxin; CTA2/B: cholera toxin subunits A2/B; CTB: cholera toxin subunit B; rCTB:recombinant CTB; i.n.: intranasal; i.g.: intragastric.

As used herein, the term "fusion protein" refers to a single contiguous protein produced by the expression of DNA sequences for one protein fused to DNA sequences encoding a different protein.

As used herein, the term "chimeric protein" refers to a fusion protein assembled with a different protein.

The present invention is directed to a composition of matter comprising a novel plasmid for use as a mucosal immunogen to prevent or inhibit the formation of dental caries. This recombinant plasmid expresses a chimeric protein which is a primary immunogen that induces long term antibody responses. Production of the chimeric protein is optimal at physiological temperatures, i.e., 37° C.

In one embodiment of the present invention, the chimeric protein immunogen is constructed by fusing a large segment of a protein antigen, e.g., Salivary Binding Protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) to the A2 subunit of cholera toxin, and assembling this with cholera toxin B subunits to form the chimeric protein. This is designated SBR-CTA2/B, or SBR-CT$^{\Delta A1}$. The latter designation refers to the deletion the A1 subunit of cholera toxin (which is the actual toxic component) from the genetic construct, and its replacement by SBR. "SBR-CTA2/B" is a shorthand molecular formula for the chimeric protein.

Initially, SBR-CTA2/B was produced in *Escherichia coli*, and the purified chimeric protein, was immunogenic by oral or intranasal administration with the generation of serum and salivary antibodies which can last for up to at least 11 months in mice. The duration of antibody responses is novel, and not predictable or expected. The establishment of regulatory T cells is in part intended to build a case for the generation of long-term memory within the mucosal immune system, because that is not 'expected' insofar as is

widely held that memory is limited in the mucosal immune system. The chimeric protein of the present invention, when expressed in attenuated *Salmonella typhimurium* produces significant increases in serum IgG and salivary IgA antibody levels after oral immunization.

A second embodiment of the present invention is the expression of SBR-CTA2/B in *Salmonella typhimurium* for delivery in a live carrier (attenuated) organism. The advantages of this construct are that there is no need to purify the product, and that a slightly different spectrum of immune responses may be obtained, with beneficial applications in some diseases.

In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin A2/B chimeric protein expressed in *E. coli*. Intragastric immunization of salivary binding protein coupled to CTB in this chimeric protein form leads to increased antigen responsive T cells.

In another embodiment of the present invention, the recombinant plasmid contains a salivary binding protein-cholera toxin$^{\Delta A1}$ chimeric protein expressed in *Salmonella typhimurium*. Oral immunization using this recombinant plasmid results in increased serum IgG responses to antigen. Oral immunization using this recombinant plasmid also resulted in increased salivary IgA antibody responses to antigen.

There are many potential uses for the technology of the present invention in mucosal vaccine development. The basic method is amenable to almost any other protein antigen that can be cloned and inserted into the construct instead of SBR. For example, a protein antigen from *Streptococcus pneumoniae* can be used to make a potential vaccine against pneumonia. Similarly, constructs from group A streptococcal proteins or a vaccine against *Helicobacter pylori* can be prepared using the methodology disclosed in the instant specification. Various applications of the present invention can be incorporated into commercial products, i.e., vaccines for the generation of immune responses that would afford protection against infections, or various modifications of the immune response. These are based on the use of CTA2/B chimeric proteins that include protein segments from a variety of microorganisms, intended for administration orally or intranasally, or possibly by other mucosal routes (e.g., rectally or intra-vaginally).

For example, one may prepare vaccines to generate immunity to the organisms responsible for dental caries, i.e., the "*mutans*" streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*). This is based on the saliva-binding region of *S. mutans* AgI/II, as described above. Secondly, one may prepare vaccines against *Streptococcus pyogenes* ("strep. throat and its sequelae including acute rheumatic fever and acute glomerulonephritis, scarlatina, streptococcal toxic shock, and other infections). Further, one may prepare: vaccines against *Streptococcus pneumoniae* (pneumococcal pneumonia, otitis media, meningitis) using sequences from pneumococcal surface proten A (PspA), vaccines against: a) *neisseria meningitidis* (meningococcal meningitis, otitis media) using neisserial surface protein A (NspA-men); b) *Neisseria gonorrhoeae* (gonorrhea) using neisserial surface protein A (NspA-gon); c) *Streptococcus pneumoniae* (pneumococcal pneumonia, otitis media, meningitis) using other pneumococcal protein antigen; d) vaccines against *Streptococcus equi* ("strangles" in horses) using a *Streptococcus equi* surface protein; e) vaccines against influenza virus *helicobacter pylori* (gastric ulcer), respiratory pathogens including *Pseudomonas aeruginosa*, f) contraceptive vaccines using zona pellucida antigens; g) vaccine against respiratory syncytial virus; h) generation of "oral tolerance" to auto-antigens (auto-immune conditions); i) vaccines against mycoplasma infections; and j) vaccines against *Staphylococcus aureus* protein A.

In yet another embodiment, the vaccine construction technology of the present invention can be used to generate immunity mediated by so-called cytotoxic T cells instead of antibodies. This methodology would have applications especially against viral infections.

Thus, the present invention is directed to a plasmid capable of replication in a host which comprises, in operable linkage:

a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin. In addition, the plasmid may further comprise DNA sequences encoding subunit B of cholera toxin fused to the A2 subunit of cholera toxin. One such preferred plasmid is pCT$^{\Delta A1}$ (deposited with ATCC, 10801 University Blvd., Manassas, Va. 20110-2209 on May 4, 1999, designation PTA-4). In another embodiment, the plasmid further comprises salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused to the A2 subunit of cholera toxin. One such preferred plasmid is designated pSBR-CTA2/B or pSBR-CT$^{\Delta A1}$ (deposited with ATCC, 10801 University Blvd., Manassas, Va. 20110-2209 on May 4, 1999, designation PTA-5).

In another embodiment, the present invention is directed to a plasmid capable of replication in a host which comprises, in operable linkage: a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin, further comprising DNA sequences encoding an antigen of interest fused to DNA sequences encoding the A2 subunit of cholera toxin. In addition, there is provided a capable of replication in a host which comprises, in operable linkage: a) an origin of replication; b) a promoter; and c) DNA sequences encoding the A2 subunit of cholera toxin further comprising DNA sequences encoding an antigen of interest fused to DNA sequences encoding the A2 subunit of cholera toxin. This plasmid may further comprises salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused to the A2 subunit of cholera toxin. The present invention also relates to chimeric proteins and fusion proteins produced by the plasmids of the present invention.

In another embodiment, the present invention is directed to an attenuated bacterial strain containing a plasmid of the present invention. In a preferred embodiment, the bacterial strain is Salmonella.

In another embodiment, the present invention is directed to a method of producing an immune response to a protein antigen of interest in an individual in need of such treatment, comprising the step of administereing to said individual a pharmacologically effective dose of a chimeric protein of the present invention. The protein may be administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, and subcutaneously. Preferably, the immune response results in the production of antibodies to the protein antigen sequence in a bodily fluid selected from the group consisting of saliva, intestinal secretions, respiratory secretions, genital secretions, tears, milk and blood. Preferably, the immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues of said individual, the development of cytotoxic T cells and immunological tolerance to the protein antigen sequence.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

One object of the present invention was to demonstrate the duration of antibody responses to the AgI/II adhesin after oral immunization of mice with SBR-CT$^{\Delta A1}$ about 1 year earlier. One group consisted of five mice previously given three doses of 100 μg of SBR-CT$^{\Delta A1}$ together with 5 μg of intact CT as an adjuvant (except for animal #5 in FIGS. 1A/1C and FIGS. 2A/2C, the mice were given the dose adsorbed on $Al(OH)_3$, which was shown to enhance serum IgG antibody responses after oral immunization).

A second group comprised three similarly treated mice with the exception that they were immunized in the absence of intact CT. A third group consisting of six mice which were sham immunized with buffer only were used as naive controls. Saliva and serum samples were collected 11 months after the last dose of the primary immunization and all three groups of mice were subsequently given 100 μg of SBR-CT$^{\Delta A1}$ by gastric intubation. CT adjuvant (5 μg) was co-administered to those mice that had also received CT during the primary immunization, and to half of the naive control animals. Samples of saliva and serum were collected again 7 days after the booster immunization and antibody responses were evaluated by ELISA on plates coated with native AgI/II and CT. Unknown antibody concentrations were calibrated against mouse immunoglobulin reference serum standards assayed simultaneously in the same microtiter plate. Results were evaluated by Student's t test using the MultiStat program (Biosoft, Cambridge, UK) with a Macintosh computer. Differences were considered significant at the $P<0.05$ level.

Substantial levels of serum IgG (FIGS. 1A and 1B) and salivary IgA (FIGS. 2A and 2B) antibodies to AgI/II and CT persisted at least until day 357, although lower than immediately after immunization (day 28), even in mice that did not receive an adjuvant dose of intact CT (FIGS. 1B and 1D plus FIGS. 2B and 2D). During the same period, the six sham-immunized mice did not develop detectable serum or salivary antibody responses, except two animals that showed trace levels of salivary IgA to AgI/II (0.15 and 0.12 % antibody/total IgA). However, the response of the sham-immunized group (0.05±0.07% antibody/total IgA) was significantly less ($P<0.05$) than the salivary responses in either of the immunized groups (0.82±0.56% antibody/total IgA (FIG. 2A) and 0.51±0.27% antibody/total IgA (FIG. 2B)). The prolonged duration of antibody responses might be explained by persisting antigen providing continuous low-level stimulation of memory cells. The mechanism of antigen persistence may involve follicular dendritic cells which bind antigen-antibody complexes via cell surface Fc receptors and slowly release them over long periods. Alternatively, the existence of molecules cross-reacting with AgI/II (or cross-reactive enterotoxins in the case of CT) cannot be ruled out, although *S. mutans* is not a natural inhabitant of the murine oral cavity.

A recall response was not observed in serum after the oral booster immunization (FIGS. 1A and 1C), as the antibody responses to AgI/II and CT before and immediately after the booster immunization were not significantly different. However one mouse (#3 in FIG. 1C) that had the lowest antibody levels to CT showed a remarkable 16-fold increase resulting in a higher final response than was observed shortly after the primary immunization. This mouse also showed an enhanced anamnestic IgG response to AgI/II which was 13 times higher than observed immediately prior to the boost (FIG. 1A). This finding suggests that anamnestic responses are not readily elicited in the presence of a relatively high persisting antibody response. As expected, naive mice developed a poor IgG antibody response to AgI/II or CT upon challenge with one dose of SBR-CT$^{\Delta A1}$.

An enhanced salivary IgA anamnestic response was not observed in these mice following the oral booster immunization, even when CT was used as an adjuvant (FIGS. 2A and 2C). Trace levels of salivary antibody to AgI/II observed in two of the six naive mice were not altered after the single booster immunization, while the remaining mice did not develop any salivary response. It appears that, after peroral immunization, the anamnestic response in the salivary glands may depend on recruitment of memory cells from the Peyer's patches or other mucosal induction sites, whereas the gut lamina propria may possess an additional source of memory represented by local memory cells that differentiate into plasma cells upon in situ activation by antigen adsorbed through intestinal epithelial cells. This might result in the memory response being manifested more readily at the gut lamina propria than at a remote effector site such as the salivary glands.

SBR represents an AgI/II adherence domain that mediates the binding of *S. mutans* to the saliva-coated tooth surfaces. S-IgA antibodies to the whole AgI/II molecule inhibit *S. mutans* adherence in vitro as well as *S. mutans* colonization and dental caries development in vivo. Since *S. mutans* infects more than 95% of the human population and caries is a common infectious disease, the continuous presence of salivary S-IgA as well as serum-derived IgG antibodies may be necessary to suppress an organism that is continually present in the oral cavity. The present invention shows that induction of long-term antibody responses is possible upon primary immunization with the SBR-CT$^{\Delta A1}$ chimeric protein. This is further supported by the finding that AgI/II-responsive T cells persist in cervical and mesenteric lymph nodes for now up to eleven months after immunization. This immunization strategy applied to other mucosal infections by linking candidate immunogens to CT$^{\Delta A1}$, may similarly elicit prolonged mucosal antibody responses.

EXAMPLE 2

Strain Construction

SBR-CT$^{\Delta A1}$ was expressed in *S. typhimurium* BRD509, an aroA$^-$ aroD$^-$ oral vaccine strain after electrotransformation with plasmids pSBR-CT$^{\Delta A1}$ and pGP-1-2 using a gene pulser (Biorad, Richmond, Calif.) set at 2.5 kV, 25 μF, and 200 Ohms. The for mer plasmid expresses SBR-CT$^{\Delta A1}$ under the inducible control of the bacteriophage T7 promoter, while the latter provides a source of T7 RNA polymerase that is temperature-regulated. Specifically, the T7 RNA polymerase is under the control of the 1 P_L promoter that is regulated by the cI857 temperature-sensitive 1 repressor. Colonies transformed with both plasmids were selected on L-agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% dextrose, 1.5% agar) supplemented with 50 μg/ml carbenicillin plus 50 μg/ml kanamycin (to select for pSBR-CT$^{\Delta A1}$ and pGP1-2, respectively). Transformants were examined for the presence of plasmids with sizes of 5.6 and 7.2 kilobases, corresponding to the size of pSBR-CT$^{\Delta A1}$ and pGP1-2, respectively.

EXAMPLE 3

Target Protein Expression and Localization

Colonies positive for both pGP1-2 and pSBR-CT$^{\Delta A1}$ were grown at 30° C. in L-broth containing the appropriate

antibiotics and target gene expression was induced at mid-log phase by a temperature shift to 42° C. After 30 minutes the cultures were returned to 30° C. and incubation was continued for an additional 90 minutes. To determine expression of SBR-CT$^{\Delta A1}$, whole-cell lysates were examined by G_{M1}-ELISA for the presence of a G_{M1} ganglioside-binding soluble protein that would react with polyclonal antibodies to CTB or AgI/II, or with a monoclonal antibody specific for the SBR of AgI/II. The insoluble pellet was then processed and possible inclusion bodies were isolated, solubilized by boiling in sodium dodecyl sulfate (SDS) buffer (the amount used was proportional to the final absorbance at 600 nm of the corresponding cultures), and samples (3 μl) analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel. To determine whether SBR-CT$^{\Delta A1}$ is transported to the periplasm, periplasmic extracts were prepared by either the spheroplast formation method or by cold osmotic shock. The extracts obtained were essentially free of cytoplasmic contamination (≦2%) as determined by assaying the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme. The total protein content of the extracts was estimated by the bicinchoninic acid protein assay method (Pierce, Rockford, Ill.) using bovine serum albumin as the standard.

EXAMPLE 4

Oral Immunization

The bacteria were grown at 30° C. to an optical density at 600 nm of 0.5–0.6, harvested by centrifugation, and resuspended in a medium consisting of 4 parts Hank's balanced salt solution (Life Technologies Inc., Grand Island, N.Y.) and 1 part sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The number of bacteria in the suspension was estimated by extrapolating from a growth curve and was confirmed by plating dilutions on L-agar plates (with or without the appropriate antibiotics) and enumerating the colonies grown after overnight incubation at 30° C. The immunizing dose (10^9 colony-forming units in 0.25 ml) was administered to 10-week old BALB/c mice by intragastric intubation using a 22-gauge feeding tube (Popper and Sons Inc., Hyde Park, N.Y.). The animals were immunized on days 0 and 49 and sampled at weekly or bi-weekly intervals. Serum was obtained from tail vein blood samples and saliva was collected after stimulation of the salivary flow by intraperitoneal injection of 5 μg carbachol. Serum IgG and salivary IgA antibodies were determined by ELISA on microtiter plates coated with AgI/II or G_{M1} ganglioside followed by CT, while total salivary IgA concentrations were assayed on plates coated with antibodies to mouse IgA. Peroxidase-conjugated antibodies to mouse IgG or IgA were used as detection reagents (Southern Biotechnology Associates, Birmingham, Ala.). The amount of antibody/immunoglobulin in test samples was calculated by interpolation on standard curves generated using a mouse immunoglobulin reference serum and constructed by a computer program based on four parameter logistic algorithms (Softmax, Molecular Devices, Menlo Park, Calif.). Results were evaluated by Student's t test and differences were considered significant at the $P<0.05$ level.

EXAMPLE 5

Results

Recombinant *S. typhimurium* BRD509 positive for both pGP1-2 and pSBR-CT$^{\Delta A1}$ was shown to produce a protein that bound the G_{M1}-ganglioside receptor and possessed CTB and AgI/II epitopes, in contrast to the original BRD509 strain or clones containing either pGP1-2 or pSBR-CT$^{\Delta A1}$ alone (TABLE I). Since the pSBR-CT$^{\Delta A1}$ encodes for the signal peptides of CTB and SBR-CTA2, it was of interest to determine whether the chimeric protein was transported into the periplasm where assembly of its components takes place.

TABLE I

G_{M1} Ganglioside-binding activity and antigenicity of soluble protein extracts[a] from recombinant *S. typhimurium* BRD509 clones

	ELISA value[b] of clone extract developed with		
Plasmid	Anti-CTB	Anti-AgI/II	Anti-SBR
None	0.017	0.003	0.001
pGP1-2	0.009	0.017	0.008
pSBR-CT$^{\Delta A1}$	0.02	0.013	0.004
pGP-1-2 + pSBRCT$^{\Delta A1}$	2.052	1.005	1.01

a = assayed at 20 μg total protein/ml; b = mean optical density at 490 nm

To examine this, a calibrated G_{M1}-ELISA standardized with purified SBR-CT$^{\Delta A1}$ was used to detect and quantify the chimeric protein in periplasmic and in whole-cell extracts under uninduced and induced (temperature shift from 30° C. to 37° C. or 42° C. for 30 minute) conditions. As shown in TABLE II, SBR-CT$^{\Delta A1}$ was found in the periplasm of *S. typhimurium* BRD509 (pGP1-2+pSBR-CT$^{\Delta A1}$) and of *E. coli* BL21(DE3) (pSBR-CT$^{\Delta A1}$) which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter. The chimeric protein was not detected in the periplasm or whole-cell lysates of a negative control clone lacking pSBR-CT$^{\Delta A1}$ (TABLE II). Although cellular location of the foreign antigen may affect the immune response, secretion of a protein into the periplasm may enhance its stability by preventing degradation.

TABLE II

Localization of SBR-CT$^{\Delta A1}$ Chimeric Protein in the Periplasmic Space

		Periplasmic fraction obtained by:		
Strain	Induction	Spheroplast formation	Osmotic Shock	Whole Cell lysate
E. coli BL21 (DE3) (pSBR-CT$^{\Delta A1}$)	IPTG[a]	271[b]	319	99.7
S. typ. BRD509 (pGP1-2)	42° C.	0	0	0
S. typ. BRD509 (pGP1-2 + PSBR-CT$^{\Delta A1}$)	None	77.3	98.5	20.2
S. typ. BRD509 (pGP1-2 + pSBR-CT$^{\Delta A1}$)	37° C.	239	314	119
S. typ. BRD509 (pGP1-2 + pSBR-CT$^{\Delta A1}$)	42° C.	119	123	53.6

a = isopropyl-β-D-thiogalactoside
b = μg SBR-CT$^{\Delta A1}$/mg protein in the extracts

Under uninduced conditions, the chimeric protein was produced at about 20 μg per mg of total soluble protein (TABLE II) or 7–9 μg per 10^9 bacteria. This finding is likely due to residual expression of the T7 RNA polymerase. Indeed, the temperature-sensitive 1 repressor on pGP1-2 does not tightly repress the 1 P_L promoter which consequently allows low-level production of the polymerase at 30° C. The amount of soluble chimeric protein increased several-fold following induction at 37° C., whereas at 42° C. the increase was minimal (TABLE II) with concomitant

accumulation of SBR-CT$^{\Delta A1}$ in inclusion bodies (FIG. 3). This suggests that at physiological body temperature (36–37° C.) production of soluble chimeric protein may be optimal.

Recombinant *S. typhimurium* expressing SBR-CT$^{\Delta A1}$ was routinely grown under uninduced conditions (30° C.) at which it showed optimal growth. Although the SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* strain does not possess a temperature-sensitive mutation, temperatures higher than 30° C. cause gradual induction of the highly-efficient T7 RNA polymerase which may interfere with gene transcription by the host RNA polymerase. Additionally overexpression of the cloned chimeric protein at 37° C. could interfere with bacterial growth. The strain was also found to be immunogenic since oral administration of 10^9 bacteria in mice resulted in serum IgG and salivary IgA antibody responses to Salmonella and native AgI/II and CT (FIGS. 4A and 4B). An oral booster immunization 7 weeks later significantly enhanced the serum IgG response to AgI/II and CT ($P<0.05$) but not to Salmonella, the response to which reached high levels even before the secondary immunization (FIG. 4A). Salivary IgA antibodies to Salmonella and the cloned antigens were detected 3 weeks after the primary immunization (FIG. 4B). The salivary response to AgI/II approached the level of 1% specific IgA antibody/total IgA but was not enhanced after the booster immunization. This is in contrast to the response against CT or Salmonella, where the increase in the response to CT or Salmonella reached statistical significance at weeks 8 or 10, respectively.

The salivary IgA response to AgI/II (~1% antibody/total IgA) after a single oral immunization with Salmonella is similar to that observed after 3 doses of 100 μg purified SBR-CT$^{\Delta A1}$ in the absence of intact CT adjuvant. The immunizing dose (10^9 bacteria) was estimated to contain 7–9 μg of chimeric protein, but this probably does not reflect the amount of SBR-CT$^{\Delta A1}$ delivered in vivo, which would largely depend on the extent of tissue colonization by Salmonella.

In the present invention, the SBR-CT$^{\Delta A1}$ chimeric protein was expressed in attenuated *S. typhimurium* and oral immunization with this recombinant strain resulted in serum IgG and salivary IgA antibody responses against Salmonella and the cloned antigens. Since the SBR segment of AgI/II plays an important role in *S. mutans* colonization, salivary IgA antibodies to SBR may confer protection against this oral pathogen.

EXAMPLE 6

Antigens

AgI/II was purified chromatographically from the culture supernatant of *S. mutans* essentially as described by Russell, et al., 1980, 28:486–493. The SBR-CTA2/B chimeric protein was constructed and expressed in *E. coli* and purified from extracts. In essence, this consisted of PCR-amplifying DNA for a 42-kDa segment encompassing the A-repeat region and some downstream sequence of AgI/II from the pac gene, ligating this in a modified pET20b(+) plasmid (Novagen, Inc., Madison Wis.) in frame with and upstream of the genes for CTA2 and CTB, and transforming the recombinant plasmid into *E. coli* BL21(DE3) cells (Novagen).

SBR polypeptide was obtained by excising the relevant DNA and religating it into unmodified pET20b(+) in order to express SBR with a 6-residue histidine sequence derived from the plasmid. This plasmid was also expressed in *E. coli* BL21(DE3), and SBR was purified from cell lysates by metal-chelation chromatography on a nickel-loaded column (Novagen), according to the manufacturer's instructions. CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell Calif.).

EXAMPLE 7

Animals and Immunization

Adult BALB/c mice of either sex, 14 to 20 weeks old, from a pathogen-free colony, were used for all experiments. Groups of 9 mice were immunized i.g. 3 times at 10-day intervals by gastric intubation of either SBR-CTA2/B (100 μg) alone, SBR-CTA2/B together with 5 μg of CT as an adjuvant, or an equimolar amount of SBR (40 μg), all given in 0.5 ml of 0.35M $NaHCO_3$. Serum and saliva samples were collected on day 0 and 10 days after each immunization for assay of antibodies by ELISA. In some experiments, subgroups of 3 mice were killed 10 days after each immunization, for the preparation of cells from PP, MLN, and spleens for T cell proliferation and flow cytometric analyses.

EXAMPLE 8

ELISA

Serum IgG and salivary IgA antibodies to AgI/II, and total salivary IgA concentrations were determined by ELISA, as described by Russell, et al., 1991, *Infect. Immun.* 59:4061–4070, on plates coated with AgI/II or anti-mouse IgA, respectively, and using goat anti-mouse IgG and IgA peroxidase conjugates as detection reagents (Southern Biotechnology Associates, Inc., Birmingham Ala.). Unknowns were interpolated on calibration curves constructed by a computer program based on four parameter logistic algorithms.

EXAMPLE 9

Preparation and Culture of Lymphoid Cells

Single-cell suspensions were obtained by teasing PP, MLN, and spleen apart with needles, and tissue debris was removed by filtering through nylon mesh. Peripheral blood mononuclear cells were obtained by centrifugation on Histopaque 1083 (Sigma Diagnostic, St. Louis, Mo.). Remaining erythrocytes were lysed in buffered ammonium chloride, the cells were washed thrice in RPMI 1640 (Cellgro) medium supplemented with 2% fetal calf serum (FCS), and were finally resuspended 10% FCS/RPMI 1640. Cells were cultured in 10% FCS/RPMI 1640 supplemented with 1 mM sodium pyruvate (Cellgro), non-essential amino acids (Cellgro), 2 mM glutamine (Cellgro), 100 U/ml penicillin-streptomycin (Cellgro), 25 mM HEPES (Sigma), and 0.01 mM 2-mercaptoethanol (Sigma).

EXAMPLE 10

Flow Cytometry

Cell marker expression on freshly isolated cells was determined by double-staining with biotinylated anti-CD4 (GK1.5) followed by avidin-phycoerythrin, and either FITC-conjugated anti-CD3 (145-2C11) or FITC-conjugated anti-CD8 (53-6.72), by incubating for 30 minutes at 4° C. in 2% FCS/Dulbecco's PBS with 0.02% NaN_3. Cells were washed, fixed in 1% paraformaldehyde overnight, and analysed on a FACStar IV flow cytometer (Becton-Dickinson).

EXAMPLE 11

Proliferation Assay

Cells from PP, MLN, and spleen were incubated at 10^5 cells/well (0.1 ml) in triplicate with a previously optimized concentration of AgI/II (0.5 μg/ml) for 5 days, and were pulsed with ^{3}[H]-thymidine (0.5 μCi/well) 8 hours before

harvesting. Uptake of ^{3}[H] was counted by liquid scintillation counter. The stimulation index was calculated as: cpm (wells with AgI/II)/mean cpm (control wells).

EXAMPLE 12

Cytokine Expression

The expression of cytokines by PP, MLN, and spleen cells after culture in vitro with or without AgI/II (0.1 μg/ml) for 24 hours was determined by a reverse-transcription and polymerase chain-reaction (RT-PCR) procedure for the amplification of cytokine mRNA. Cells (5–7×10^6) were harvested from the cultures, washed thoroughly, and then lysed in 350 μl of lysing buffer for isolation of RNA using the RNeasy kit (Qiagen Inc., Chatsworth Calif.). RNA was redissolved in 40 μl of diethyl pyrocarbonate-treated water, and 2 μl samples were added to 18 μl of RT mixture (Perkin-Elmer, Foster City Calif.) containing 1× PCR buffer, 5 mM $MgCl_2$, 1 mM of each deoxyribonucleoside triphosphate, 1 U/ml RNase inhibitor, 2.5 U/ml Moloney murine leukemia virus reverse transcriptase, and 2.5 mM Oligo $d(T)_{16}$. Mixtures were overlaid with 50 μl of light mineral oil and incubated in a thermal cycler (Perkin-Elmer) for 15 minutes at 42° C., 45 minutes at 37° C., 5 minutes at 99° C., and 5 minutes at 4° C. After reverse transcription, 80 μl of PCR mixture (Perkin-Elmer) was added to each tube to give final concentrations of 25 U/ml AmpliTaq DNA polymerase, 0.15 μM 5' primer, 0.15 μM 3' primer, 2 mM $MgCl_2$, and 1× PCR buffer II. Primers specific for murine IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and β-actin were obtained from Clontech Laboratories Inc. (Palo Alto, Calif.) or the Oligonucleotide Synthesis Core Facility of the UAB Comprehensive Cancer Center, and their specificity was verified by means of RT-PCR on RNA extracted from mitogen-stimulated mouse spleen cells. After heating at 95° C. for 2 min, cDNA was amplified for 35 cycles consisting of: 45s at 94° C., 3 minutes at 72° C., and 2 minutes at 60° C. The products of amplification were analysed by 2% agarose gel electrophoresis, revealed by ethidium bromide staining, and photographed by UV transillumination. The results were scored according to the presence of a band of appropriate molecular size: −, no detectable band; ±, very faint or uncertain band; +, clearly detectable band; ++, very strong band.

EXAMPLE 13

Statistical Methods

Quantitative results were evaluated by Student's t test, by means of MultiStat (Biosoft, Ferguson Mo.) on a Macintosh computer. Antibody data were transformed logarithmically to normalize their distribution and homogenize the variances.

EXAMPLE 14

Antibody Responses

I.g. immunization of mice with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT incrementally induced serum IgG and salivary IgA antibodies measured against whole AgI/II (FIGS. 5A and 5B). Immunization with SBR alone resulted in weak but statistically significant ($P<0.001$ at all intervals) serum IgG antibody responses, and modest salivary IgA antibodies that were significantly elevated above background only after the second and third immunization ($P<0.001$ and $P<0.01$, respectively). Administration of the SBR-CTA2/B chimeric protein generated significantly greater serum IgG responses ($P<0.001$), and co-administration of CT as an adjuvant further enhanced both the level and the earlier development of serum IgG antibodies. Salivary IgA antibodies also tended to be elevated by immunization with SBR-CTA2/B chimeric protein especially when given with CT as adjuvant. However, because of variation between animals, statistical significance was attained only after 2 doses given with CT. Nevertheless, the general pattern of results was in accordance with expectations based on responses to AgI/II alone or chemically conjugated to CTB, and administered i.g. without or with CT adjuvant. Total salivary IgA concentrations also increased in all animals during the immunization period, from 2.13±0.61 μg/ml in unimmunized animals to 5.92±0.64 μg/ml after 3 immunizations, but there were no significant differences between the immunization groups.

EXAMPLE 15

T Cell Proliferative Responses

To show whether T cells capable of proliferating in vitro in response to stimulation with AgI/II had been induced by the first, second, or third i.g. dose, groups of 3 mice were killed 10 days after a first, second, or third immunization with each immunogen preparation, and mononuclear cells from PP, MLN, and spleens were cultured with or without AgI/II. Incorporation of ^{3}[H]-thymidine expressed as stimulation indices revealed that AgI/II responsive cells were elicited in the lymphoid tissues associated with the intestine, incrementally with the number and form of the immunogen doses (FIG. 6). PP and MLN cells taken from mice given 2 or 3 doses of SBR or of SBR-CTA2/B alone showed modest proliferative responses to AgI/II in vitro (stimulation indices in the range 2.4–3.2; 5.44 for PP from mice given 3 doses of SBR-CTA2/B), whereas PP and MLN cells from mice immunized with SBR-CTA2/B plus CT adjuvant showed proliferative responses after one dose (stimulation indices 2.3 and 3.6, respectively), and greater responses after 2 or 3 doses (stimulation indices 3.1–6.1). The proliferative responses of PP and MLN cells were different: MLN cells responded similarly to (or less than) PP cells when taken from mice immunized with SBR or SBR-CTA2/B, but showed greater responses to AgI/II in vitro when taken from mice given AgI/II-CTA2/B plus CT. Spleen cells generally did not respond to stimulation with AgI/II in vitro (stimulation indices <2), except for those taken from mice immunized once with SBR-CTA2/B plus CT (stimulation index=2.8). Cells from the PP, MLN, or spleens of unimmunized mice did not proliferate in response to AgI/II in vitro (stimulation indices 1.2–1.5).

EXAMPLE 16

T Cell Surface Marker Analysis

To elucidate the nature of the T cell responses to i.g. immunization, cells freshly isolated from PP, MLN, spleen, or peripheral blood of mice immunized once, twice, or three times with the different immunogens were analyzed by flow cytometry for the proportion of cells expressing T cell markers CD3 (all T cells), CD4 (T helper phenotype), or CD8 (T suppressor/cytotoxic phenotype). The results are shown in FIG. 7. Among PP cells, there was an increase in the proportion of total T cells after each immunization that was most noticeable in animals immunized with SBR-CTA2/B or SBR-CTA2/B plus CT. This increase was mostly in the CD4$^+$ T helper population, whereas the CD8$^+$ T suppressor/cytotoxic population remained small. The MLN cell populations remained more stable, except in the case of cells from mice immunized with SBR-CTA2/B plus CT in which the CD4$^+$ population increased with the number of immunizations. MLN generally, however, contained more T cells of both phenotypes than PP, regardless of immunization status. Peripheral blood cells tended to show the greatest increases in the proportion of CD4$^+$ T cells after

immunization, especially with SBR-CTA2/B plus CT, although these numbers must be interpreted with caution because of the small numbers of cells obtained. Spleen cells showed modest increases in the proportions of $CD4^+$ T cells after immunization in all groups.

alone revealed type 1 (IFN-γ and IL-2) as well as type 2 (IL-4) cytokine responses upon stimulation in vitro, whereas cells from the same organs of mice immunized with SBR-CTA2/B chimeric protein revealed IL-4 but little or no type 1 cytokine response.

TABLE III

Cytokine expression in PP, MLN and spleen cell cultures of mice immunized with SBR, SBR-CTA2/B or SBR-CTA2/B + CT

		IFN-γ			IL-2			IL-4			IL-5		
Immunization[a]	Culture[b]	P	M	S	P	M	S	P	M	S	P	M	S
SBR	Control	–[c]	–	–	–	±	–	±	–	–	+	+	+
	+AgI/II	+	+	+	++	+	++	++	–	++	+	+	+
SBR-CTA2/B	Control	–	–	–	–	±	–	+	±	+	+	+	+
	+AgI/II	–	±	+	–	+	++	+	+	++	+	+	+
SBR-CTA2/B + CT	Control	–	–	+	–	±	+	±	±	+	+	+	+
	+AgI/II	–	+	++	–	±	++	+	+	++	+	+	+

[a]mice were immunized thrice at 10 day intervals and organs collected 3 days after the last immunization.
[b]cells were cultured in vitro for 24 hours without (control) and with AgI/II (0.1 ug/ml)
[c]cytokine mRNA expression detected by RT-PCR and scored according to the presence of ethidium bromide-stained band of appropriate molecular size;
–; no detectable band
±; very faint/uncertain band;
+, clear band; and
++, very strong band.
P = PP cell cultures,
M = MLN cell cultures;
S = spleen cell cultures.

EXAMPLE 17

Cytokine Expression

To elucidate the pattern of expression of cytokines, PP, MLN, and spleen cells were taken from mice immunized three times with SBR, SBR-CTA2/B, or SBR-CTA2/B plus CT 3 days after the last dose, cultured in vitro for 24 hours with or without AgI/II, and examined for the presence of mRNA for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 by RT-PCR. After culture with AgI/II, PP, MLN, and spleen cells from mice immunized with SBR alone revealed mRNA for IFN-γ and IL-2, but only PP and spleen cells also revealed IL-4 mRNA, whereas IL-5 mRNA was detectable in all cell cultures regardless of stimulation (TABLE III). PP cells from mice immunized with SBR-CTA2/B, without or with CT adjuvant, did not reveal mRNA for IFN-γ or IL-2, even after culture with AgI/II, and MLN cells from these animals revealed variable IFN-γ and IL-2 mRNA responses. However, PP, MLN, and spleen cells revealed IL-4 mRNA particularly after stimulation with AgI/II, whereas all cultures were positive for IL-5 mRNA. Likewise, mRNA for IL-6 and IL-10 was found in all cell cultures, regardless of immunization or in vitro stimulation. There was an increase in IFN-γ and IL-2 expression (in response to stimulation with AgI/II in vitro) in PP, MLN, and spleen cells from mice immunized 3 times with SBR alone relative to cells from mice immunized twice. Likewise, spleen cells from mice immunized 3 times with SBR-CTA2/B (without or with CT) showed increased AgI/II-specific expression of IFN-γ, IL-2, and IL-4 relative to twice-immunized mice. Cells from unimmunized mice did not respond in culture with AgI/II by the expression of IFN-γ, IL-2, and IL-4 mRNA above that revealed in control cultures, except that spleen cells showed weak evidence of IFN-γ expression on culture with AgI/II. Thus, PP and MLN cells from mice immunized with SBR

Lymphocytes taken from the PP and MLN of mice immunized i.g. with SBR, or SBR-CTA2/B without or with CT as adjuvant were capable of proliferating in vitro when stimulated with AgI/II, showing a similar overall pattern of T cell responses to the different regimens and stages of immunization as the serum and salivary antibody responses. Immunization with SBR alone induced the lowest proliferative responses in PP and MLN cells, and this was reflected also in little change in the proportions of $CD4^+$ and $CD8^+$ T cells in these organs. Moreover, the pattern of cytokine expression in the cells from PP and MLN of these mice suggested a mixed type 1 and type 2 helper activity, possibly governed by Th0 cells. Coupling SBR to CTB in the form of the SBR-CTA2/B chimeric protein enhanced its immunogenicity with respect to T cell responses in PP and MLN, and the addition of CT as an adjuvant further elevated these responses. Furthermore, the cytokine expression pattern in PP and MLN cells from mice immunized with SBR-CTA2/B (with or without CT) indicated that T cell help was skewed towards Th2 activity. However, the finding of IL-5, IL-6, and IL-10 mRNA in cell cultures regardless of antigen stimulation in vitro is not readily explained in these terms, but may indicate constitutive expression of these cytokines or their continued expression ex vivo after immunization. It is also possible that Il-6 and IL-10 mRNA were derived from macrophages present in the cell cultures, although these would be largely adherent and unlikely to be harvested along with the lymphocytes.

The proportions of $CD4^+$ T cells in PP increased after each additional dose of these immunogen preparations, but a corresponding increase was seen in MLN cells only from mice immunized with SBR-CTA2/B chimeric protein and CT adjuvant. The finding that these T cell responses occurred in PP and MLN as early as after the first immunization, at least with SBR-CTA2/B, showed that antigen-sensitized T cells were elicited before IgA antibody responses became elevated in the effector sites of mucosal

immunity such as salivary glands. The responses in MLN and PP were different, as significant proliferative responses and increased proportions of $CD4^+$ cells during the course of immunization were developed in MLN cells only when CT was used as an adjuvant, and moreover MLN from all mice contained higher proportions of T cells of both phenotypes than corresponding PP. The proportion of $CD8^+$ cells was higher in MLN than in PP, but as it was not reduced by the administration of CT as an adjuvant, it appears that the enhanced AgI/II-specific proliferation in MLN cells from mice given CT is not due to inhibition of $CD8^+$ suppressor cells by CT. The spleen, a non-mucosal lymphoid organ, displayed little or no response in terms of antigen-specific proliferating T cells, despite the considerable elevation of serum IgG antibodies especially when SBR-CTA2/B was given together with CT adjuvant. This is consistent with the relatively modest numbers of specific antibody-secreting cells found in the spleen after i.g. immunization with AgI/II chemically conjugated to CTB and given with CT. Throughout these experiments, although the mice were immunized with SBR or SBR-CTA2/B chimeric protein representing residues 186 to 577 of AgI/II, both antibody and T cell responses could be detected with intact AgI/II. This implies that SBR retains conformational structure similar to that of the corresponding part of the whole AgI/II molecule, and that both are processed similarly by antigen-presenting cells.

These responses are in accordance with the concept of the common mucosal immune system, and the dissemination of antigen-sensitized T and B cells from the inductive sites such as PP, through the MLN that drain the lymph flow from the small intestine, and thence into the circulation prior to relocating in the effector sites of mucosal immunity, including the salivary glands. Thus, i.g. immunization with SBR, especially when coupled to CTB in the form of a chimeric protein, leads to the appearance of antigen-responsive T cells in both PP and MLN. Because few cells were recoverable from blood, it was not practically possible to trace the appearance of such cells in the circulation, although this has been well documented in humans. The transient circulation of specific antibody-secreting cells, predominantly of the IgA isotype, approximately one week after mucosal immunization has been demonstrated in human and animal systems. Curiously, perhaps, it appears that the peak of circulating antigen-specific T cells occurs after the peak of circulating antibody-secreting cells, and an increased proportion of $CD4^+$ T helper cells was found in the peripheral blood of mice 10 days after the second or third dose of SBR-CTA2/B, especially if CT was also given as an adjuvant. Cytokine-secreting T cells occur in effector sites of mucosal immunity, such as the salivary glands.

CT has been shown to enhance T helper responses in intestinal tissues, and particularly the Th2 subset that is held to promote high levels of serum IgG and mucosal IgA antibody responses. Type 2 cytokine production by antigen-specific T cells in nasal-associated lymphoid tissue and the draining cervical lymph nodes of mice immunized intranasally, as well as in PP and MLN of mice immunized i.g., with AgI/II conjugated to CTB were also found. CT is known to deplete selectively $CD8^+$ intraepithelial lymphocytes and while the functions and migratory potential of these cells are incompletely understood, any such effect within inductive sites such as the PP would also serve to elevate the proportion of $CD4^+$ T cells. However, although the proportion of $CD8^+$ cells declined slightly in some tissues, this appeared to occur concomitantly with an increase in the number of $CD3^+$ cells, in particular the $CD4^+$ subset. Whether CTB itself can serve as an adjuvant in the absence of intact CT has been controversial. Synergism between CTB and CT has been demonstrated and most commercially available, non-recombinant preparations of CTB contain small amounts of intact CT that may be sufficient to show this effect. The genetically constructed SBR-CTA2/B chimeric protein, in which the toxic CTA1 subunit has been deleted, is clearly able to induce mucosal and circulating antibodies without the necessity for additional CT. The adjuvant activity of CT may be closely linked to its toxicity which is a function the ADP-ribosyltransferase activity of the A1 subunit. Adjuvanticity of the related *Escherichia coli* heat-labile enterotoxin can be dissociated from toxicity. Fusion proteins of CTB directly coupled to other antigenic peptides have been constructed, but the conformation of CTB and its ability to form G_{M1}-binding pentamers tend to be disrupted by peptides longer than approximately 12 amino acid residues and moreover, their mucosal immunogenicity seems to be limited in the absence of additional CT. These limitations do not apply to SBR-CTA2/B chimeric protein, in which a large 42 kDa segment of protein is fused to the CTA2 subunit which couples it noncovalently to the CTB pentamer to preserve its G_{M1} ganglioside-binding activity. The enhanced enteric immunogenicity of SBR-CTA2/B chimeric protein, even in the absence of CT, is advantageous for an oral vaccine, as recombinant CTB has been shown to be a safe and effective immunogen in humans.

I.g. immunization with SBR, especially when genetically coupled to CTB to enhance both mucosal and circulating antibody responses, induces T cell responses in the gut-associated lymphoid tissues such as PP and MLN. Furthermore, these T cell responses occur after one or two doses of immunogen, earlier than the antibody responses, and include increased proportions of $CD4^+$ T helper cells. The responses are enhanced by, but are not dependent upon, the addition of CT as an adjuvant.

EXAMPLE 18

Expression of SBR in *S. typhimurium*

The pac gene segment encoding the SBR region (1.2 kilobases [kb]) was removed from pSBR-$CT^{\Delta A1}$ by restriction digestion with the NcoI and XhoI endonucleases, and purified after agarose gel electrophoresis of the digest and extraction using the QIAEX gel extraction kit (Qiagen, Chatsworth, Calif.). The pET20b(+) expression vector (3.7 kb; Novagen, Madison, Wis.) was similarly digested by NcoI and XhoI, dephosphorylated by calf intestinal alkaline phosphatase and purified by gel extraction. The pac segment was then ligated into pET20b(+) in frame with the 3' end of the pelB leader sequence (required for the transport of cloned polypeptides into the periplasm) and the resulting ligation product, designated pSBR, was introduced into *S. typhimurium* BRD509 (pGP1-2) (6) by means of electroporation using a gene pulser (Biorad, Richmond, Calif.) set at 2.5 kV, 25 μF, and 200 Ohms. Transformed colonies were selected on L-agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% dextrose, 1.5% agar) supplemented with 50 μg/ml carbenicillin and 50 μg/ml kanamycin (to select for pSBR and pGP1-2, respectively). Transformants were examined for the presence of two plasmids with sizes of 4.9 and 7.2 kb, corresponding to the size of pSBR and pGP1-2, respectively. Both plasmids were required for the expression of SBR since its transcription in pSBR is under the control of the bacteriophage T7 promoter and pGP1-2 provides a source of T7 RNA polymerase. Expression of the SBR polypeptide in transformants containing both plasmids was induced at mid-log phase by a shift from 30° C.–37° C., and production of SBR was confirmed by western immunoblot-

ting of cell lysates using antibodies to the native AgI/II molecule (FIG. 8B). Target gene induction in this system is temperature-regulated because the T7 RNA polymerase is under the control of the 1 P_L promoter that is regulated by the cI857 temperature-sensitive 1 repressor (FIG. 8A).

EXAMPLE 19

Estimation of Recombinant Protein Production

To determine the amount of the SBR polypeptide produced by *S. typhimurium* (pGP1-2+pSBR-CT$^{\Delta A1}$) (6) and *S. typhimurium* (pGP1-2+pSBR) (FIG. 8B), a calibrated "sandwich" ELISA standardized with purified rSBR was performed using cell lysates obtained by sonication. This quantitative ELISA was repeated four times using independent cultures which were grown at 30° C. and subsequently processed for the immunizations (see "Immunizations" below). The construction of standard curves and the interpolation of the unknowns was performed by means of a computer program based on four-parameter logistic algorithms (Softmax/Molecular Devices, Menlo Park, Calif.). For the ELISA, rabbit anti-mouse IgG followed by a mouse monoclonal IgG antibody to SBR served as the coating reagents while peroxidase-conjugated rabbit polyclonal antibodies to native AgI/II was used for detection of bound protein. SBR used as standard was purified from cell lysates by metal-chelation chromatography on a nickel-charged column (Novagen), according to the manufacturer's instructions. The affinity of SBR for nickel arises from a 6-residue histidine sequence (at its C-terminal end) which was derived from the pET-20b(+) expression vector. Recovery of SBR from the column was achieved by elution with imidazole. The purity of the SBR preparation was verified by SDS-PAGE and its protein content was estimated by the bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.) using BSA.

A similar approach was used to quantify the SBR-CT$^{\Delta A1}$ chimeric protein with the exception that the plates were coated with G_{M1} ganglioside (Calbiochem, La Jolla, Calif.). This G_{M1}-ELISA was standardized with SBR-CT$^{\Delta A1}$ purified. Briefly, the chimeric protein was isolated from whole-cell extracts by size-exclusion chromatography on a Superose 12 HR 16/50 column (Pharmacia-LKB, Piscataway, N.J.) followed by anion-exchange chromatography on a Mono Q column (Pharmacia-LKB). Results are expressed as %SBR or SBR-CT$^{\Delta A1}$ per total soluble protein in the lysates.

EXAMPLE 20

Immunizations

Overnight cultures of recombinant *S. typhimurium* BRD509 expressing SBR-CT$^{\Delta A1}$ or SBR were diluted 1:100 in L-broth containing 50 μg/ml of kanamycin and 50 μg/ml of carbenicillin and grown at 30° C. with shaking and aeration until A_{600nm} reached 0.5–0.55. The bacteria were recovered by centrifugation and resuspended in a medium consisting of 4 parts Hank's balanced salt solution (Life Technologies Inc., Grand Island, N.Y.) and 1 part sodium bicarbonate (7.5% solution; Mediatech, Washington, D.C.). The number of bacteria in the suspension was estimated by extrapolating from a growth curve and was confirmed by plating dilutions of the bacterial inoculum on L-agar plates (with or without the appropriate antibiotics) and enumerating the colonies grown after overnight incubation at 30° C.

BALB/c mice, 10 to 12 weeks old, from a pathogen-free colony, were used for oral and i.n. immunization studies performed according to NIH guidelines and protocols approved by the UAB Institutional Animal Care and Use Committee. An oral dose containing 10^9 CFU in 0.25 ml was administered to groups of 5–6 mice by intragastric (i.g.) intubation using a 22-gauge feeding tube (Popper and Sons Inc., Hyde Park, N.Y.) The mice were immunized 1 to 3 times in a period of 6 days. A single booster immunization with 10^{10} CFU was given 15 weeks later. For i.n. immunizations, groups of 6 mice were inoculated 3 times (in a period of 6 days) with 10^8 CFU in a volume of ~20 μl which was slowly applied in the external nares by means of a micropipettor. A single booster i.n. immunization with 10^9 CFU was performed 15 weeks later. In another experiment, groups of 3 mice received a single primary immunization (10^{10} CFU for i.g. or 10^9 CFU for i.n. delivery) followed by a booster immunization with the same dose 15 weeks later. An age-matched, unimmunized control group consisting of 5 mice was also included to monitor background antibody levels during the course of the studies.

EXAMPLE 21

Sampling and Quantification of Antibody Responses

Serum was obtained by centrifugation of blood samples collected from the lateral tail vein with heparinized capillary pipettes. Preimmune samples were obtained 1 day before the immunizations and subsequent to immunizations collections were made 3, 5, and 7 weeks later, one day before the booster immunization (week 15), and at biweekly intervals thereafter (weeks 17 and 19). Saliva samples were collected at the same times as serum by means of a pipettor fitted with a plastic tip after stimulation of salivary flow by i.p. injection of 5 μg carbachol (Sigma Chemical Company, St. Louis, Mo.). Fecal extracts were prepared by vortexing 3 fecal pellets from each mouse in 600 μl extraction buffer (PBS containing 0.02 % azide, 1% BSA, 1 mM PMSF, and 5 mM EDTA). The extracts were subsequently centrifuged and the supernatants obtained were assayed for total IgA levels (see below) and were adjusted to contain 100 μg of total IgA per ml ("standardized" fecal extracts) by adding an appropriate volume of extraction buffer.

The levels of isotype-specific antibodies from serum, saliva, or fecal extracts, and total salivary or intestinal IgA were determined by ELISA on microtiter plates coated with native AgI/II (chromatographically purified from *S. mutans* culture supernatants), G_{M1} followed by CT (List Biological Laboratories, Campell, Calif.), formalin-killed cells of *S. typhimurium* BRD509, or goat anti-mouse IgA. The plates were developed with the appropriate peroxidase-conjugated goat anti-mouse Ig isotype (IgG for serum samples and IgA for secretion samples) and o-phenylenediamine substrate with H_2O_2. IgG 1 or IgG2a antibody responses were assayed using peroxidase-conjugated IgG subclass-specific antibodies. All antibodies used for ELISA were purchased from Southern Biotechnology Associates, Inc., Birmingham, Ala. The concentration of antibodies/total Ig in test samples was calculated by interpolation on standard curves generated using a mouse Ig reference serum (ICN Biomedicals, Costa Mesa, Calif.) and constructed by a program based on four parameter logistic algorithms (Softmax/Molecular Devices).

EXAMPLE 22

Statistical Analysis

Results were evaluated by Student's t test by means of the Multistat program (Biosoft, Cambridge, UK) on a Macintosh computer. Differences were considered significant at the $P<0.05$ level. antibody data were logarithmically transformed to normalize their distribution and homogenize the variances. The data were finally retransformed and presented as geometric means ×/÷ SD.

EXAMPLE 23

Recombinant Protein Production by the Salmonella Clones

Using a "sandwich" ELISA calibrated with purified rSBR, it was determined that *S. typhimurium* (pGP1-2+pSBR-CT$^{\Delta A1}$) and *S. typhimurium* (pGP1-2+pSBR) produced similar amounts of the SBR polypeptide (Table IV). Slightly higher levels of SBR were detected in the lysates from the pSBR-containing clone than in the extracts from the clone expressing the SBR-CT$^{\Delta A1}$ chimeric molecule, but the difference was not statistically significant. This might have resulted from the presence of CTA2/B which could sterically interfere with the recognition of the SBR component of the chimeric protein by the antibodies used in the "sandwich" ELISA. As expected, G_{M1}-ELISA calibrated with purified SBR-CT$^{\Delta A1}$ detected a G_{M1}-binding protein possessing SBR epitopes only in lysates from the pSBR-CT$^{\Delta A1}$-containing clone (TABLE IV). The expression level of SBR-CT$^{\Delta A1}$ in the lysates was approximately 2–3% of the total soluble protein (TABLE IV). These results validate the appropriateness of comparing the capacity of these two clones to induce antibody responses to the SBR of AgI/II.

Analysis of serum samples obtained from i.n. immunized mice for anti-AgI/II responses, confirmed the trend of higher responses in mice immunized with *S. typhimurium* expressing SBR than SBR-CT$^{\Delta A1}$ seen in i.g. immunized animals (FIG. 11A). In this case the differences in anti-AgI/II responses between the clones were statistically significant except for the responses on week 17 (FIG. 11A). This trend did not seem to correlate with the anti-Salmonella responses induced by i.g or i.n. immunization. In fact, the anti-vector responses appeared to be higher for the SBR-CT$^{\Delta A1}$-producing clone; FIGS. 10A and 11B. The kinetics as well as the magnitude of the serum IgG responses to Salmonella and the cloned Ags after i.n. immunization (FIGS. 11A and 11B) were comparable to those after i.g. immunization (FIGS. 9A, 9B, 9C and 10A) despite that the number of Salmonellae given by the i.n. route was lower by one order of magnitude.

In the above experiments, preimmune serum samples (from 10- to 12-week old mice) did not contain detectable antibodies to AgI/II, CT, or Salmonella (corresponding data points at week 0 in FIGS. 9A, 9B, 9C, 10A, and 11A and 11B, represent the detection limit of the ELISA). Furthermore, 27 week-old unimmunized controls did not show substantial antibody levels to AgI/II or CT (≦1.2 μg/ml) but they did develop a relatively weak response (compared to immunized animals) against Salmonella (17.8×/÷1.6 μg/ml, n=5), probably because of cross-reactions with related gram-negative bacteria of their normal flora.

TABLE IV

Production of recombinant protein by *S. typhimurium* (pGP1-2 + pSBR and *S. typhimurium* (pGP1-2 + pSBR-CT$^{\Delta A1}$) clones

	Elisa Method			Protein Amount[a]	
Assay:	Coating	Develop.	Exp.	pSBR	pSBR-CT$^{\Delta A1}$
SBR	SBR-MAb	AgI/II-PAb	No. 1	1.68	1.18
			No. 2	1.33	1.23
			No. 3	1.51	1.07
			No. 4	1.13	1.14
			Mean ± SD	1.39 ± 0.21	1.16 ± 0.07
SBR-CT$^{\Delta A1}$	Gm1	AgI/II-PAb	No. 1	0**	2.03
			No. 2	0	3.05
			No. 3	0	3.49
			No. 4	0	2.13
			Mean ± SD	0	2.68 ± 0.71

[a]amount of recombinant protein in cell lysate of clones containing either pSBR or pSBR-CT$^{\Delta A1}$;
* = % SBR polypeptide/total soluble protein in cell lysates;
** = % SBR-CT$^{\Delta A1}$ chimeric protein/total soluble protein in cell lysates.

EXAMPLE 24

Serum IgG Antibody Responses

Oral immunization of mice with the *S. typhimurium* clones expressing SBR or SBR-CT$^{\Delta A1}$ resulted in dose-dependent primary serum IgG responses to AgI/II which were significantly enhanced after a single booster dose (FIG. 9A). Although the responses to AgI/II induced by i.g. administration of either *S. typhimurium* clone (i.e., expressing SBR alone or linked to CTA2/B) were not statistically different, a trend was observed towards higher serum IgG responses after two or three primary immunizations with *S. typhimurium* expressing SBR alone than with the clone expressing the SBR-CT$^{\Delta A1}$ chimeric protein (FIGS. 9B and 9C).

The response to the Salmonella vector was essentially similar with increasing number of doses (one to three) given during primary immunization (data shown for the groups which received two primary doses; FIG. 10A). As expected, serum IgG antibodies to CT were induced after immunization with the clone expressing the chimeric SBR-CT$^{\Delta A1}$ molecule but not with the clone expressing SBR alone (FIG. 10A). As with anti-AgI/II responses, the serum IgG response to CT was also significantly elevated following the booster immunization (FIG. 10A).

In these studies antibody responses to the SBR of AgI/II were detected using native AgI/II as the coating agent in the ELISA, as recombinant vaccines should be able to induce responses against the antigen expressed by the pathogen. On a limited number of samples, the influence of coating with purified rSBR on the magnitude of the detected responses was also determined. ELISA with serum samples tested on plates coated with either rSBR or AgI/II showed that the antibody response to rSBR was about 2 times higher than to AgI/II (2.15×/÷1.49, n=13), implying substantial antigenic and possibly structural similarities between rSBR and the corresponding region (residues 186 to 577) of the whole AgI/II. Moreover, the antibody response against the CTA2/B component of the chimeric protein was about 3 times higher (3.34×/÷1.10, n=6) when detected with native CT bound to G_{M1} ganglioside-precoated plates than when CT was directly coated on microtiter plates.

EXAMPLE 25

IgG2a/IgG1 Profile for Vector and Carried Antigens

To determine the subclass distribution of serum IgG antibody responses, samples from mice orally immunized with SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* were analyzed for levels of IgG2a and IgG1 antibody responses which are indicative of a Th type-1 or Th type-2 response, respectively. Serum IgG antibodies to whole Salmonella belonged predominantly to the IgG2a subclass (IgG2a/IgG1>10) but a mixed IgG2a and IgG1 response pattern (IgG2a/IgG1~1) was observed for the cloned Ags, SBR and CTA2/B (TABLE V). Analysis of samples from a study in which mice were orally immunized with purified SBR-CT$^{\Delta A1}$, revealed predominant IgG1 responses to SBR and CTA2/B, indicating that immunization with the *S. typhimurium* vector shifted the responses towards the IgG2a subclass (TABLE V). In the case of the anti-SBR responses the shift was statistically significant. In terms of the IgG2a/IgG1 ratio, the response to SBR was not influenced by the route of immunization (i.g. or i.n.) or the presence or absence of CTA2/B (clones expressing SBR-CT/(^{A1} or SBR alone).

TABLE V

Profile of IgG2a/IgG1 antibody responses in serum after oral immunization with SBR-CT$^{\Delta A1}$ expressed in *S. typhimurium* or administered as purified immunogen

Immunization with	antibody response to:	IgG2a/IgG1*	No. of mice IgG2a/IgG>1
S. typhimurium vector (n = 16)	SBR	1.02 ± 2.26**	8/16
	BTA2/B	1.21 ± 2.6	10/16
	Salmonella	11.2 ± 2.21	16/16
Purified SBR-CT$^{\Delta A1}$ (n = 6)	SBR	0.3 ± 1.94	0/6
	CTA2/b	0.48 ± 1.73	1/6

* = ration obtained following quanfication of subclass-specific antibody levels
** = values are the geometric mean x/(SD of the IgG2a/IgG1 ratios of individual mice.

EXAMPLE 26

Salivary IgA Antibody Responses

Oral immunization of mice with recombinant Salmonella vector, one to three times, resulted in the induction of increasingly higher salivary IgA antibody responses to AgI/II (FIGS. 12A, 12B, and 12C). A single oral booster immunization resulted in augmented antibody levels in the groups immunized one or two times during priming, whereas the secondary response in the groups immunized three times for priming was slightly lower than the peak primary response at the time measured (FIG. 12C). All groups of mice displayed significantly higher antibody levels four weeks after the secondary immunization than immediately before the booster immunization. Interestingly, although the anti-AgI/II responses induced by the SBR- and the SBR-CT$^{\Delta A1}$-expressing clones were generally not significantly different (except for the responses of the groups immunized three times for primary immunization where differences reached statistical significance after the boosting (FIG. 12C)), they showed the opposite trend than that observed in the serum IgG responses, i.e., the presence of CTA2/B appeared to enhance the salivary IgA response to AgI/II. In contrast, the salivary IgA anti-vector responses induced by the two Salmonella clones were very similar (data shown for the groups which were given two primary doses; FIG. 10B). IgA antibodies to CT in saliva were detected after immunization with the SBR-CT$^{\Delta A1}$-expressing clone only, and were significantly elevated by secondary immunization (FIG. 10B). The salivary IgA responses to Salmonella and the cloned Ags after i.n. administration of the recombinant *S. typhimurium* (FIGS. 13A, 13B and 13C) displayed similar characteristics as after i.g. immunization and were comparable in magnitude despite the use of lower doses. The secondary response to CT and AgI/II reached significantly higher levels than the primary response, two and four weeks following the booster immunization, respectively. When saliva samples were normalized for total IgA content, samples from 27-week-old unimmunized mice showed similar levels of "background" antibody activity to the test Ags as the preimmune samples of immunized animals reported in FIGS. 10B, 12A, 12B, 12C, 13A, 13B, and 13C.

The finding that anti-AgI/II responses tended to be higher in saliva but lower in serum when mice were immunized with the SBR-CT$^{\Delta A1}$-expressing clone than with the clone producing SBR alone, was further supported by data from additional groups of mice. These mice were given a single immunization of 10^{10} CFU by the i.g. or 10^9 CFU by the i.n. route, boosted with the same dose 15 weeks later, and displayed the above mentioned trend regardless of the route of administration (FIGS. 14A and 14B). In the same experiment, the mice immunized with a single i.g. dose of 10^{10} CFU showed higher anti-AgI/II responses in serum and saliva than mice which received a single i.g. dose of 10^9 CFU (FIGS. 9A and 12A), but equal or slightly lower responses than mice given 3 i.g. doses of 10^9 CFU (FIGS. 9C and 12C).

EXAMPLE 27

Intestinal IgA Responses

IgA anti-AgI/II responses were also detected in fecal extracts from i.g. immunized mice (FIGS. 15A, 15B, and 15C). The kinetics of the responses induced by the two recombinant *S. typhimurium* clones also showed some trend for higher anti-AgI/II responses when the SBR antigen was co-expressed with CTA2/B (FIGS. 15A, 15B, and 15C). This trend was less pronounced than seen in saliva (FIGS. 12A, 12B, and 12C), and it did not show statistical significance except for two time points, i.e., FIG. 15A, at week 15 with groups given 1 primary dose, and, FIG. 15B, at week 19 with groups given 2 primary doses. At the same time, the anti-Salmonella response appeared to be higher in the case of the clone producing SBR alone (FIG. 10C), suggesting that the relatively high anti-AgI/II responses in the case of the SBR-CT$^{\Delta A1}$ clone may be related to the co-expression of SBR and CTA2/B. Intestinal IgA responses to Salmonella and the cloned Ags were also induced after immunization by the i.n. route (FIGS. 16A and 16B). As in the case of saliva, fecal samples from 27 week-old unimmunized mice showed similar levels of "background" antibody activity against the test Ags with the preimmune samples of immunized animals, but the background activity against CT appeared to be higher compared to that against AgI/II or even Salmonella (FIGS. 10C, 15A, 15B, 15C, 16A, and 16B).

Using a temperature-regulated expression system engineered in avirulent *S. typhimurium*, the present invention demonstrated high levels of antibodies against the cloned heterologous Ags in serum and mucosal secretions after oral or i.n. immunization. Expression of recombinant immunogens in this system was activated under in vivo conditions (37° C.), since at 37° C. target protein induction was shown to be optimal. This system was used to investigate whether the non-toxic A2/B moiety of cholera toxin can act as a Salmonella-cloned adjuvant when co-expressed with the SBR protein antigen. For this purpose, a *S. typhimurium* clone expressing SBR alone and a similar clone expressing the SBR-CT$^{\Delta A1}$ chimeric protein were used, which were found to produce similar levels of the SBR polypeptide. The amount of chimeric protein produced by the SBR-CT$^{\Delta A1}$

clone (2.68% of total soluble protein) was consistent with the estimated SBR levels (1.16%) expressed by the same clone, since SBR comprises 39% of the whole chimeric molecule by weight.

Quantitative analysis of serum samples showed that the IgG responses to SBR were generally lower after immunization with the SBR-CT$^{\Delta A1}$-expressing *S. typhimurium* than with the clone expressing SBR alone. Although this may suggest intramolecular antigenic competition as observed within the IgG molecule, i.e., higher responses are induced to the Fab fragment when Fab is injected alone than when the whole IgG molecule is used for immunization, analysis of responses in secretions revealed an opposite trend. Comparing the mucosal IgA antibody levels to AgI/II induced by immunization with the two *S. typhimurium* clones at each time point examined (56 time-points including all groups from both i.g. and i.n. immunization; FIGS. **12A, 12B, 12C, 13B, 14B, 15A, 15B, 15C, 16A,** and **16B**), the response in the case of the clone expressing SBR linked to CTA2/B was higher on 51 occasions (91%). In contrast, the serum IgG response was higher in 83% of the occasions (31/36) in the case of the other clone, i.e., the one expressing SBR alone (FIGS. **9A, 9B, 9C, 11B,** and **14A**). One can speculate that CTA2/B may have a dual influence on anti-SBR responses arising from its dual role as an immunogen and as an immunoenhancing agent. As an immunogenic component of the SBR-CT$^{\Delta A1}$ chimeric molecule, CTA2/B may tend to depress the immune response to SBR through antigenic competition, and as a mucosal adjuvant it may tend to potentiate anti-SBR responses. This dual effect might have differentially influenced the observed mucosal and systemic responses if CTA2/B is able to provide better help for antibody production in mucosal inductive sites than in systemic compartments. Presumably, Salmonella-expressed CTA2/B can be delivered to both mucosal and systemic inductive sites because of the ability of the vector to colonize mucosal lymphoid tissues and to disseminate to systemic tissues. Interestingly, in an immunization study with influenza virus administered mucosally or systemically in the absence or presence of CTB, the adjuvant effect of CTB on antiviral antibody responses was found to be more pronounced after i.n. than after subcutaneous or i.p. immunization. These findings cannot be attributed to quantitative differences (equal doses were given by all routes and in the case of i.n. immunization the amount actually absorbed may be even less than that injected for systemic administration) but rather to a CTB adjuvant effect which is possibly influenced by the particular microenvironment where CTB acts.

Th1 or Th2 cells induce antigen-specific B cells to selectively produce IgG2a or IgG1 antibodies, respectively. Salmonella (as well as other intracellular microorganisms) generally induces a Th1-type response characterized by high levels of IFN-γ and IgG2a antibodies. The serum IgG response to the *S. typhimurium* vector displayed a high (>10) IgG2a/IgG1 ratio. Moreover, a mixed IgG2a and IgG1 response (IgG2a/IgG1≈1) was induced against Salmonella-expressed SBR, although a predominant IgG1 response to SBR was observed after oral immunization with purified SBR-CT$^{\Delta A1}$. These data also suggest that the type of response to a Salmonella-delivered protein antigen was not entirely determined by the vector but is also influenced by inherent properties of the cloned antigen.

The mechanisms for inducing intestinal IgA responses to orally administered vaccines have been extensively studied. Although less is known regarding responses induced after i.n. immunization, several mechanisms can be offered for the observed intestinal IgA responses following i.n. administration of recombinant Salmonella in mice. Antigenic stimulation of the nasal-associated lymphoid tissues which show anatomical similarities with the Peyer's patches in the gut (e.g., lymphoid follicles covered by M cells) may result in the dissemination and homing of lymphoid cells to remote mucosal effector sites, including the intestinal lamina propria, in a fashion analogous to stimulation of the GALT. Moreover, a portion of the Salmonella inoculum may have been swallowed by the mice resulting in direct stimulation of the GALT. To minimize this possibility, a relatively small volume was slowly applied to the external nares (~10 μl per nostril). If these mice did swallow some Salmonella organisms, the number would be relatively small compared to the oral dose given to i.g. immunized mice, i.e., 10 times higher than the i.n. dose. Alternatively, *S. typhimurium* could access the GALT by dissemination from nasal lymphoid tissues. Immunization by the i.n. route was generally as effective as by the i.g. route, despite using lower doses.

Besides a remarkable secondary IgG response in serum against AgI/II and CT, a pronounced secondary salivary IgA response was induced against the cloned Ags after i.n. and i.g. (especially when two primary doses were given) immunization, suggesting induction of immunological memory. Enhanced salivary IgA anamnestic responses to SBR or CTA2/B were not observed previously in mucosal immunization experiments using purified SBR-CT$^{\Delta A1}$ or the whole AgI/II molecule chemically conjugated to native CTB. Because of concerns regarding the efficacy of repeated use of Salmonella as a carrier for various heterologous Ags, it was of interest that boosting of mucosal IgA and serum IgG antibody responses was induced after i.g. booster immunization of mice with a pre-existing intestinal IgA response to the Salmonella vector.

In summary, despite the requirement for genetic coupling of CTA2/B to SBR to induce substantial anti-SBR responses after mucosal immunization with purified immunogen, expression of SBR alone in an avirulent *S. typhimurium* vector was sufficient to induce high levels of antibodies in serum and mucosal secretions. The finding that the immunogenicity of Salmonella-delivered SBR was not significantly dependent on co-expression of CTA2/B, suggests that in oral immunization with purified SBR-CT$^{\Delta A1}$ targeting of SBR to the GALT via G_{M1} receptors on the overlying antigen-sampling M cells, may constitute an important immunoenhancing mechanism. The requirement for this mechanism, which would also reduce the exposure of SBR to proteases in the gut lumen, is bypassed by the Salmonella vector because of its tropism for the GALT, where SBR will eventually be delivered. The current system can be modified so that CTA2/B can find application as a Salmonella-cloned adjuvant, especially for Ags that are poor immunogens when delivered by this live antigen-delivery system.

EXAMPLE 28

Intranasal Immunization of Rats with AgI/II, AgII, and SBR Chemical/Genetic Conjugates

Fischer rats, 19 days old, were used for intranasal immunization studies performed according to NIH guidelines and protocols approved by the UAB Institutional Animal Care and Use Committee. The animals were immunized 3 times at 14-day intervals with 50 μg of the appropriate inunuogen (see group designations), with or without an adjuvant amount (1 μg) of cholera toxin (CT), in a volume of 50 μl which was slowly applied in the external nares by means of a micropipettor.

For sampling and quantification of antibody responses, serum was obtained by centrifugation of blood samples

collected from the retroorbital plexus with heparinized capillary pipettes. Saliva samples were collected by means of a Pasteur pipette after stimulation of ovary flow by intraperitoneal injection of 10 μg carbachol. Samples were obtained 1 day before the immunizations and 2 weeks after the last immunization. The levels of isotype-specific antibodies from serum and saliva, and total salivary IgA were determined by ELISA on microtiter plates coated with native AGI/II, recombinant SBR, AgII, G_{MI} followed by CT, or goat anti-rat IgA. The plates were developed with the appropriate peroxidase-conjugated goat anti-rat immunoglobulin isotype (IgG for serum samples and IgA for saliva samples) and o-phenylenediamine substrate with H_2O_2. The concentration of antibodies/total immunoglobulin in test samples was calculated by interpolation on standard curves generated using a rat immunoglobulin reference serum and constructed by a computer program based on four parameter logistic algorithms.

The results that follow are presented as μg/ml of specific serum IgG antibody or as % specific salivary IgA antibody/total IgA. Antibody data were logarithmically transformed to normalize their distribution and homogenize the variances. The data were finally retransformed and presented as geometric means ×/+ standard deviation for ease of interpretation. The presented data are from samples obtained 2 weeks after the last immununization. Specific antibodies in preimmunune samples were not detectable.

Immunization groups 1, 2, and 3 are controls, groups 4 and 5 were immunized with SBR-CTA2/B chimeric protein (SBR-CT^{AA}, shown as CHIM) without (group 4) or with (group 5) CT as adjuvant, and groups 6–11 were immunized with chemical conjugates of SBR, AgII, or AgI/II coupled to recombinant CTB, without or with CT as an adjuvant.

Antibody responses were assayed against the intact AgI/II (FIGS. 17 and 21), as well as against SBR (FIGS. 18 and 22), and AGII (another part of AGJM distinct from the part containing SBR; FIGS. 19 and 23). Responses against CT (FIGS. 20 and 24) are given for comparison, since the immunogens as well as the adjuvant (CT) where used also induce responses to CT. Since CT is regarded as the most potent mucosal immunogen, these comparisons serve to put the magnitude of responses to SBR or Ag I in perspective.

In all instances, responses (measured against SBR, AgI/II, or AGII) to the various immunogens given without CT adjuvant were undetecable; the use of CT as adjuvant was necessary to obtain responses in these rats (Fischer strain). This finding is in marked contrast to all previous results obtained in mice (BALB/c strain), in which antibody responses to SBR-CTA2/B chimeric protein or to AgI/II conjugated chemically to rCTB given intranasally were generated in the absence of CT adjuvant. The reason for this difference of response is not clear, and it is not known if this reflects the particular strain of rat used, or is typical of rats as compared to mice. Reports of studies performed on human subjects indicate that humans respond well to intranasal or oral immunization with recombinant CTB, i.e., more like mice than rats. The following is a comparison of responses to the different immunogens for animals immunized with CT as adjuvant.

Both SBR (in the form of either the chimeric immunogen, group 5; or the chemical conjugate, group 7) and AGII (group 9) as well as the intact AgI/II (group 9) induced antibodies detectable against AgI/II in serum (IgG, FIG. 17) and in saliva (IgA, FIG., 21). The intact AgI/II and AgII (serum only) tended to induce the strongest responses measured in this way. However, SBR (in either form) induced the strongest responses measurable against SBR (FIGS. 18 and 22) whereas AgII failed to induce responses to SBR. Conversely, AgII induced responses measurable against AgII (FIGS. 19 and 23) but SBR did not. Despite the fact that the use of CT as an adjuvant also induced very strong responses to itself (FIGS. 20 and 24), the responses to SBR, AgI/II, and AgII compare well with the responses to CT.

Therefore, the chimeric immunogen, SBR-CTA2/B, is very effective for inducing serum IgG and salivary IgA antibodies to SBR, which also react with the parent antigen, AgI/II. This may be advantageous, because SBR was selected as a part of the, AgI/II molecule that appears to be functionally important for the adherence of *Streptococcus mutans* to tooth surfaces. Immunization with AgI/II appears to induce antibodies most strongly against the AgII part of the molecule, yet earlier work indicated that antibodies to AgII might not be protective against *S. mutans*-induced dental caries. The advantage of using genetically constructed chimeric immunogens may therefore include the ability to direct an antibody response to a functionally important part of tbe antigen molecule that is otherwise less immunogenic than parts of the the molecule that may be functionally less important. Such a finding may be of considerable importance in vaccine development, since surface molecules of microorganisms that have functional activity in pathogenesis may have evolved structures that divert host immune responses away from the more sensitive parts of the molecule.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

What is claimed is:

1. A method of producing an immune response by administration of an attenuated strain of bacteria, wherein said attenuated bacteria express an antigen of interest as a fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter; and,

c) DNA sequences encoding the antigen of interest, wherein said DNA sequences are fused in frame to the A2 subunit of cholera toxin.

2. The method of claim 1, wherein salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II) is the antigen of interest fused to the A2 subunit of cholera toxin.

3. The method of claim 1, wherein said plasmid further comprises DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein to facilitate assembly of a chimeric protein.

4. The method of claim 3, wherein said plasmid is pCT^{AA}.

5. The method of claim 3, wherein salivary binding protein (SBR) from *Streptococcus mutans* surface protein (Ag I/II) is the antigen of interest fused to the A2 subunit of cholera toxin.

6. The method of claim 5, wherein said plasmid is pSBR-CT^{AA1} (alternatively designated pSBR-CTA2/B).

7. The method of claim 3, wherein said attenuated bacterial strain is administered by a route selected from the group consisting of orally, intranasally, intrarectally, intravaginally, intramuscularly, and subcutaneously.

8. The method of claim 3, wherein said immune response results in the production of antibodies to the protein antigen sequence in a bodily fluid selected from the group consisting of saliva, intestinal secretions, respiratory secretions, genital secretions, tears, milk and blood.

9. The method of claim 3, wherein said immune response is selected from the group consisting of development of antigen-specific T cells in the circulation and tissues of said individual, the development of cytotoxic T cells and immunological tolerance to the protein antigen sequence.

10. An attenuated Salmonella strain, wherein said Salmonella strain expresses a chimeric fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter;

c) DNA sequences encoding a fusion protein of the antigen of interest fused in frame with the A2 subunit of cholera toxin; and,

d) DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein of the antigen of interest and A2 cholera toxin subunit to facilitate assembly of a chimeric protein.

11. An attenuated Salmonella strain, wherein said Salmonella strain expresses a chimeric fusion protein from a plasmid which comprises in operable linkage:

a) an origin of replication;

b) a promoter;

c) DNA sequences encoding a fusion protein of salivary binding protein (SBR) from *Streptococcus mutans* surface protein (AgI/II) fused in frame to the A2 subunit of cholera toxin; and,

d) DNA sequences encoding subunit B of cholera toxin for coexpression with said fusion protein of saliva binding protein and A2 cholera toxin subunit to facilitate assembly of a chimeric protein.

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